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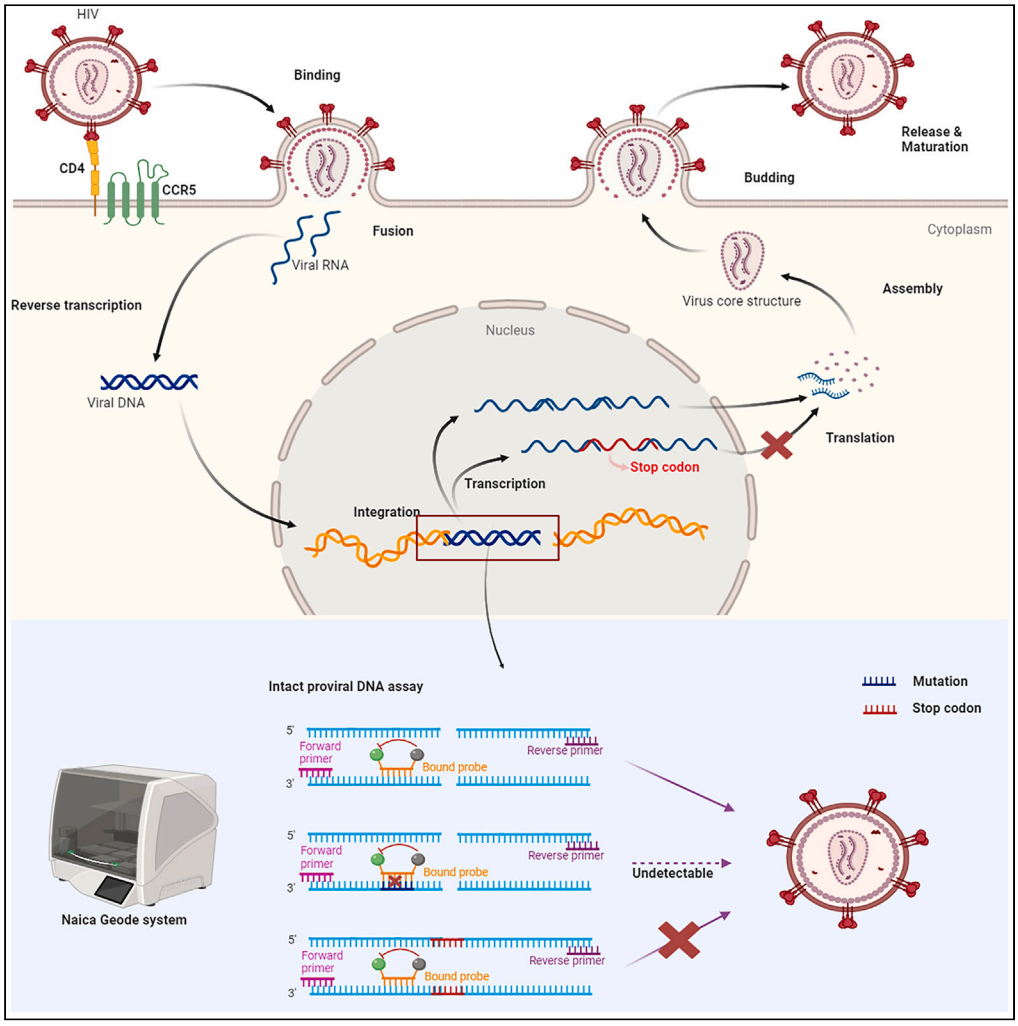

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Highlights

The high diversity of HIV-1 reduces the accuracy of IPDA

IPDA-B cannot evaluate the change of HIV-1 latency reservoirs in patients infected with CRF01_AE

One type of IPDA cannot detect all HIV-1 subtypes

Mutations in *env* gene outside the probe binding site impact the detection efficiency of IPDA

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HIV-1 env gene mutations outside the targeting probe affects IPDA efficiency

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SUMMARY

The intact proviral DNA assay (IPDA) based on droplet digital PCR was developed to identify intact proviral DNA and quantify HIV-1 latency reservoirs in patients infected with HIV-1. However, the genetic characteristics of different HIV-1 subtypes are non-consistent due to their high mutation and recombination rates. Here, we identified that the IPDA based on the sequences features of an HIV-1 subtype could not effectively detect different HIV-1 subtypes due to the high diversity of HIV-1. Furthermore, we demonstrated that mutations in *env* gene outside the probe binding site affect the detection efficiency of IPDA. Since mutations in *env* gene outside the probe binding site may also lead to the formation of stop codons, thereby preventing the formation of viruses and ultimately overestimating the number of HIV-1 latency reservoirs, it is important to address the effect of mutations on the IPDA.

INTRODUCTION

It is a major barrier that a stable latent form of HIV-1 persists in $CD4^+$ T cells even during suppressive effective antiretroviral therapy (ART).[1–3] Once ART is discontinued, HIV-1-infected $CD4^+$ T cells contribute to high levels of viremia.[4] The latent reservoir is defined as cells carrying intact HIV-1 proviruses, while cells carrying defective proviruses are not latent reservoirs.[5] Defective proviruses are defined as those that contain overt fatal defects such as deletions and/or hypermutation.[6,7] Intact proviral DNA, accounting for 7% of the total HIV-1 DNA, is the source of replication-competent virus in HIV-1-positive people.[8] Therefore, it is important to develop assays that distinguish intact proviruses from the overwhelming majority of defective proviruses, which is critical for the clinical treatment of people infected with HIV-1.

The gold standard of HIV-1 latent reservoir measurement is the quantitative viral outgrowth assay (QVOA) for infected cells releasing replication-competent virus after a single round of T cell activation *in vitro*.[1,4] However, some studies have shown that the QVOA underestimates reservoir size because not all cells with intact proviruses are induced by a single round of T cell activation.[9–11] Sequencing of near-full-length proviral clones allows accurate quantification of latent reservoirs, but the method is expensive and not scalable for clinical situations.[12,13] The intact proviral DNA assay (IPDA) is a novel approach for quantifying the HIV-1 latent reservoir using multiplex digital droplet PCR.[7,8,14] The Robert F. Siliciano team firstly designed the IPDA that simultaneously detects two HIV-1 regions containing the packaging signal (Ψ) near the 5′ end of HIV-1 and the Rev-response element (RRE) containing two apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G (APOBEC3G) consensus sites (TGGG) within the envelope (*env*), which can jointly identify >90% of defective proviruses.[7]

To date, the IPDA has been designed primer/probe based on the sequence characteristics of subtype B, hereinafter referred to as IPDA-B, because the majority of HIV cure research is conducted in resource-rich countries for people infected with HIV-1 subtype B.[7,8,14] However, the most prevalent epidemic subtype is not HIV-1 subtype B in some areas, such as Asia and sub-Saharan Africa.[15–19] In China, a large number of circulating recombinant forms (CRFs) were identified. CRF01_AE and CRF07_BC have become the major HIV-1 subtypes, accounting for 70.73% of all HIV-1 subtypes from 2017 to 2020, whereas B accounts for only 2.49%.[20] Moreover, there is up to 30% sequence diversity among different HIV-1 subtypes.[21] Some authors have reported that HIV-1 polymorphisms may contribute to IPDA failure.[22] We therefore investigated the HIV-1 diversity characterization that influences the effectiveness of the IPDA. Moreover, we further investigated the effect of

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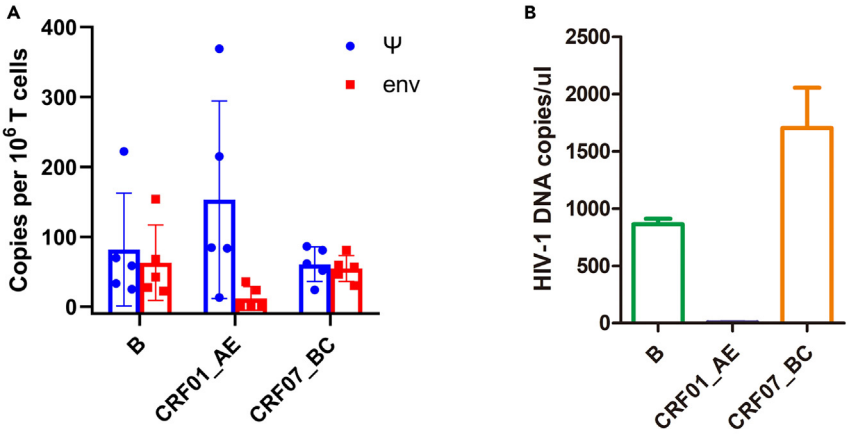

Figure 1. Verification of the effectiveness of IPDA-B in detecting different HIV-1 subtypes

(A) DNA from PBMCs infected with different HIV-1 subtype viral isolates, including B, CRF01_AE, and CRF07_BC, was quantified with IPDA-B.

(B) IPDA-B detected plasmids containing the env from different HIV-1 subtypes.

See also Table S1.

high mutation frequencies within the *env* gene outside the probe binding site on IPDA, which provides an important basis for optimizing the effectiveness of the IPDA.

RESULTS

Effect of HIV-1 diversity on IPDA-B

We applied IPDA-B to detect the latency reservoirs of 24 patients infected with different HIV-1 subtypes, including subtypes CRF01_AE, CRF07_BC, and B. The use of crystal readers for data analysis (Stilla Technologies) has been described in previous studies.[23] The results showed that IPDA-B could detect intact (i.e., Ψ and *env* double positive) proviruses in people infected with subtypes B and CRF07_BC, but IPDA-B was unable to effectively detect the *env* probe binding site of HIV-1 CRF01_AE (Figure 1A). Then, we constructed plasmids containing *env* from different HIV-1 subtypes, including subtypes CRF01_AE, CRF07_BC, and B, diluted all plasmids to a concentration of 10 ng/μL, and detected these plasmids using IPDA-B. The average copy number per microliter is 5.55 for CRF01_AE, 863.93 for subtype B, and 1,703.75 for CRF07_BC (Figure 1B). The results indicated that IPAD-B could not effectively detect subtype CRF01_AE, but it could detect subtypes CRF07_BC and B.

The sequence of the *env* probe binding site of CRF01_AE was not consistent with that of other subtypes

Over 3,500 HIV-1 *env* sequences were collected from the Los Alamos National Library HIV Database (www.hiv.lanl.gov), including CRF01_AE (n = 960), CRF07_BC (n = 734), and B (n = 1,807). We analyzed the binding site of *env* primers and probes for different HIV-1 subtypes. There was no significant difference in the binding site of *env* primers, which did not affect the amplification efficiency of *env* primers (Figure 2A). However, there were differences in the binding site of the *env* probe. T is the second base of the binding site of the *env* probe for subtype CRF01_AE, and the 13th base is A/G. C/T is the second base of the binding site of the *env* probe for subtypes B and CRF07_BC, and the 13th base of the two subtypes is G (Figure 2B).

Design of a probe for detecting the reservoir of latent HIV-1 CRF01_AE proviruses

Based on the sequence characteristics of CRF01_AE, we designed a probe (sequence: CTTTGGGTTCTTAGGATC) for the detection of CRF01_AE (IPDA-CRF01_AE). Using IPDA-B and IPDA-CRF01_AE, we detected HIV-1 DNA copies in 10 ng/μL CRF01_AE recombinant plasmids and the latency reservoir in individuals infected with HIV-1 CRF01_AE. IPDA-CRF01_AE detected an average of 8,087.48 copies/uL, while IPDA-B only detected an average of 7.11 copies/uL (Figure 3A). Moreover, IPDA-B only detected 395.27 copies per 10^6 T cells, but IPDA-CRF01_AE detected 1,295.58 copies per 10^6 T cells (Figure 3B). Therefore, these results suggested that IPDA-B could not effectively detect CRF01_AE, but IPDA-CRF01_AE could effectively detect the number of latency reservoir in individuals infected with HIV-1 CRF01_AE.

IPDA-CRF01_AE effectively monitors changes in the latency reservoir of individuals infected with CRF01_AE

Some studies have shown that after ART treatment, the latency reservoir in HIV-1-positive people will significantly decrease in the short term.[24,25] We separately collected peripheral blood mononuclear cells (PBMCs) from patients infected with HIV-1 CRF01_AE prior to treatment and six months after ART treatment. We analyzed the latency reservoir with IPDA-B and IPDA-CRF01_AE. The latency reservoir of HIV-1 CRF01_AE-infected patients was 319.50 copies per 10^6 T cells before treatment and 218.03 copies per 10^6 T cells after six months treatment by

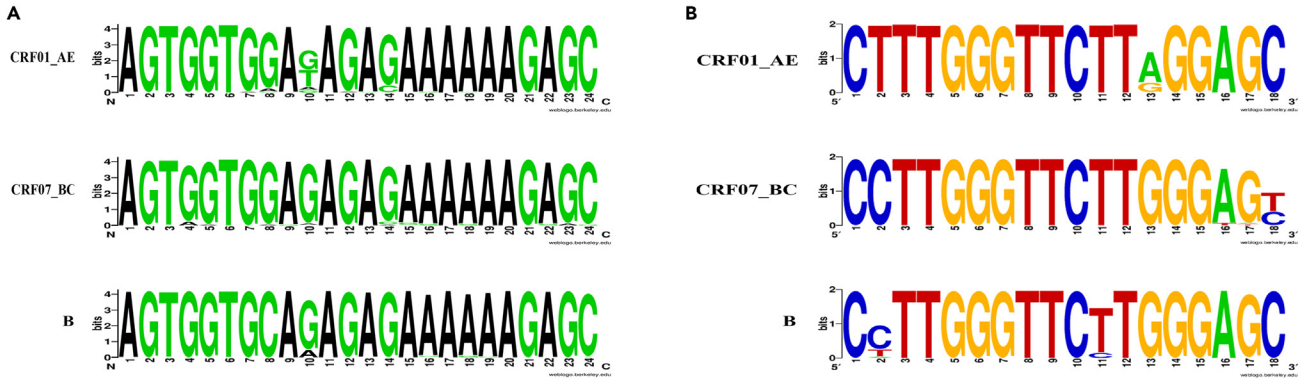

Figure 2. Analysis of the binding site of the env primer and probe for different HIV-1 subtypes, containing CRF01_AE (*n* = 960), CRF07_BC (*n* = 734), and B (*n* = 1807)

(A) Env forward primer binding site for different HIV-1 subtypes.

(B) Env probe binding site for different HIV-1 subtypes. The plots were generated using the WebLogo tool.

IPDA-B (Figure 4A). However, the latency reservoir of HIV-1 CRF01_AE-infected patients was 1,822.33 copies per 10^6 T cells before treatment and 884.33 copies per 10^6 T cells after six months treatment by IPDA-CRF01_AE (Figure 4B). Our results suggested that IPDA-CRF01_AE could not only accurately evaluate the latency reservoir of patients infected with HIV-1 CRF01_AE, but also reflect the significant decrease in the latency reservoir of patients after treatment.

Mutations within *env* outside the probe binding site affect the validity of IPDA

Env has the highest level of diversity among all HIV-1 proteins.[26,27] Moreover, there are many mutations in *env*, and viral diversity within a host increases over time in HIV-1-infected people,[28,29] which may cause inactivation of HIV-1. First, we applied IPDA-B and IPDA-CRF01_AE to detect the latency reservoir in people infected with subtype CRF07_B and CRF01_AE, respectively. Then, we extracted DNA from these positive PBMC samples, and *env* sequences were amplified, which were inserted into pcDNA3.1(+) plasmids, and conducted monoclonal screening. The positive plasmids were sequenced to obtain env gene information. Subtypes of the gene were identified by phylogenetic analysis (Figure S1). Then, the positive clones were packaged with HIV-1 backbone plasmids to form pseudoviruses, and TZM-bl cells were used to detect their infectivity. We analyzed the sequences characteristic of the env probe binding site. First, the frequency of each amino acid in the sequences isolated from the same patients was analyzed, and then all sequences from different patients also were analyzed. The results showed the sequence of env probe binding sites remained highly consistent, regardless of HIV-1 CRF01_AE (Figure 5A) or CRF07_BC (Figure 5B), and there were no mutations that could affect probe detection. Even though the sequence of env probe binding sites remained highly consistent, our results indicated that a large proportion of these CRF01_AE pseudoviruses were not infectious (Figure 5C). Similarly, a large proportion of CRF07_BC pseudoviruses were not infectious neither (Figure 5D). Moreover, we analyzed the sequences characteristic of *env* gene outside the probe binding site. The results showed that a large number of mutations were found in *env* gene outside the probe binding site. 11 types of mutations in CRF01_AE led to the formation of stop codons, thereby preventing the formation of viruses (Figure 5E). There were also 7 types of mutations in CRF07_BC that caused the formation of stop codons and prevented the formation of viruses (Figure 5F).

DISCUSSION

The intact HIV-1 proviral reservoir produces a large amount of infectious HIV-1, which is a huge challenge to cure HIV-1. The IPDA effectively detects intact HIV-1 proviral reservoirs and evaluates the size of latency reservoirs. There are multiple subtypes of HIV-1, and the genetic differences between subtypes are significant. Moreover, mutations in *env* increase over time in HIV-1-infected people, leading to HIV-1 inactivation. Therefore, high-frequency mutations affect the efficiency of IPDA detection. First, our findings demonstrated that IPDA-B cannot effectively detect HIV-1 CRF01_AE. Then, we designed IPDA-CRF01_AE for detecting CRF01_AE and further investigated the ability of IPDA-CRF01_AE to detect an intact HIV-1 proviral reservoir. However, the accumulation of genetic mutations leads to many variants in HIV-1-infected patients as infection continues. These mutations affect the accuracy of IPDA detection because mutations outside the probe's binding site may also inactivate HIV-1.

The prevalence of HIV-1 varies among countries and regions, with subtype B mainly prevalent in Europe and North America,[30–32] and CRF01_AE in Asia, which accounted for over 34.63% of the cases in China.[15–17,20] Robert F. Siliciano first used the IPDA-B method to detect the intact HIV-1 proviral reservoir and designed it based on the sequence of HIV-1 B.[7] Herein, we used IPDA-B to detect CRF01_AE, which could not effectively detect CRF01_AE but could detect CRF07_BC. There were significant differences in *env* sequences between B and CRF01_AE. Therefore, we designed probes based on CRF01_AE to effectively detect CRF01_AE. Several studies have revealed a reduction in the number of latency reservoirs in HIV-1 patients after ART treatment.[24,25] Then, we applied the IPDA to test the HIV-1 latency reservoir prior to treatment and 6 months after treatment in HIV-1 patients. The results showed that IPDA-CRF01_AE could effectively reflect the

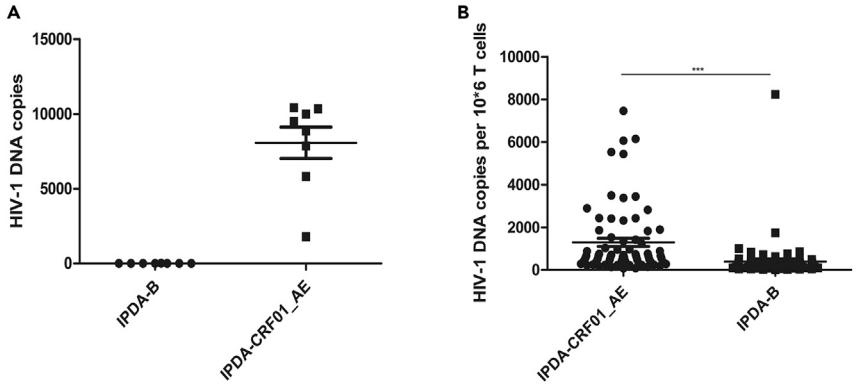

Figure 3. Comparison of the efficiency of IPDA-B and IPDA-CRF01_AE in HIV-1 CRF01_AE detection

(A) IPDA-B and IPDA-CRF01_AE detected plasmids containing the env of HIV-1 CRF01_AE.

(B) IPDA-B and IPDA-CRF01_AE detected latency reservoirs in patients infected with HIV-1 CRF01_AE. The statistical difference between the IPDA-B and IPDA-CRF01_AE was determined with Student's t test (***: $p < 0.001$).

changes in the latency reservoir in patients infected with CRF01_AE. Therefore, IPDA-CRF01_AE is more effective than IPDA-B in evaluating the size of the latency reservoir in patients infected with CRF01_AE. Previous data suggested that the RRE is affected by the APOBEC3G,[7] which leads to high-frequency mutations and HIV-1 inactivity.[33,34] Some articles also reported that *env* is prone to mutations, and changes in some bases within *env* lead to the loss of HIV-1 infection ability.[35–37] The IPDA targets only a segment of *env*, and mutations in other regions may also cause the inactivation of HIV-1, so we applied monoclonal screening techniques to obtain a larger number of *env* sequences isolated from HIV-1 patients and constructed pseudoviruses. The *env* probe binding sites of these pseudoviruses were consistent, but there were many mutations in *env* gene outside the probe binding site, causing the formation of stop codons that prevent the formation of viruses. This result suggested that the application of IPDA was influenced by mutations within *env* gene outside the probe binding region, which may also contribute to HIV-1 inactivation. Therefore, the IPDA needs to be further optimized for detecting the HIV-1 latency reservoirs.

In conclusion, the IPDA ineffectively detects different HIV-1 subtypes due to the high diversity of HIV-1; therefore, more subtypes of IPDA need to be designed for detecting different HIV-1 subtypes. Moreover, even if the *env* probe binding sites of different subtypes of pseudoviruses are consistent, mutations in *env* gene outside the probe binding site also lead to the formation of stop codons, preventing the formation of infectious HIV-1. Therefore, IPDA can overestimate the HIV-1 latency reservoirs, which affects the evaluation of clinical treatment.

Limitations of the study

This experiment has some limitations because HIV-1 pseudovirus is composed of only the env sequence and does not involve other HIV-1 genes. Therefore, the pseudovirus cannot reflect the effect of genetic mutations in other genes on the IPDA. Second, we did not obtain more HIV-1 subtypes for analysis of the broad spectrum of the IPDA.

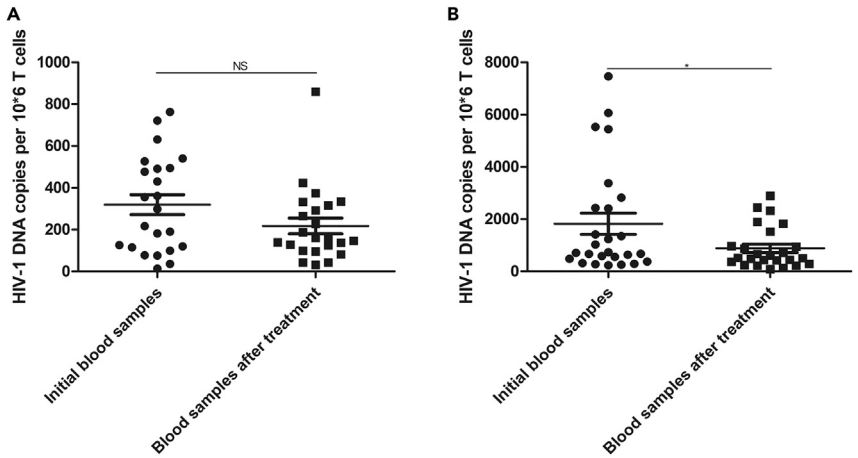

Figure 4. Using IPDA-B and IPDA-CRF01_AE, we detected changes in the latency reservoir in individuals infected with CRF01_AE

(A) The use of IPDA-B supported detection of latency reservoirs in individuals infected with CRF01_AE before treatment and after ART treatment.

(B) The use of IPDA-CRF01_AE supported detection of the latency reservoir in individuals infected with CRF01_AE before treatment and after ART treatment. The statistical difference between the Initial blood samples and Blood samples after treatment was determined with Student's t test (ns: no significance; *: $p < 0.05$).

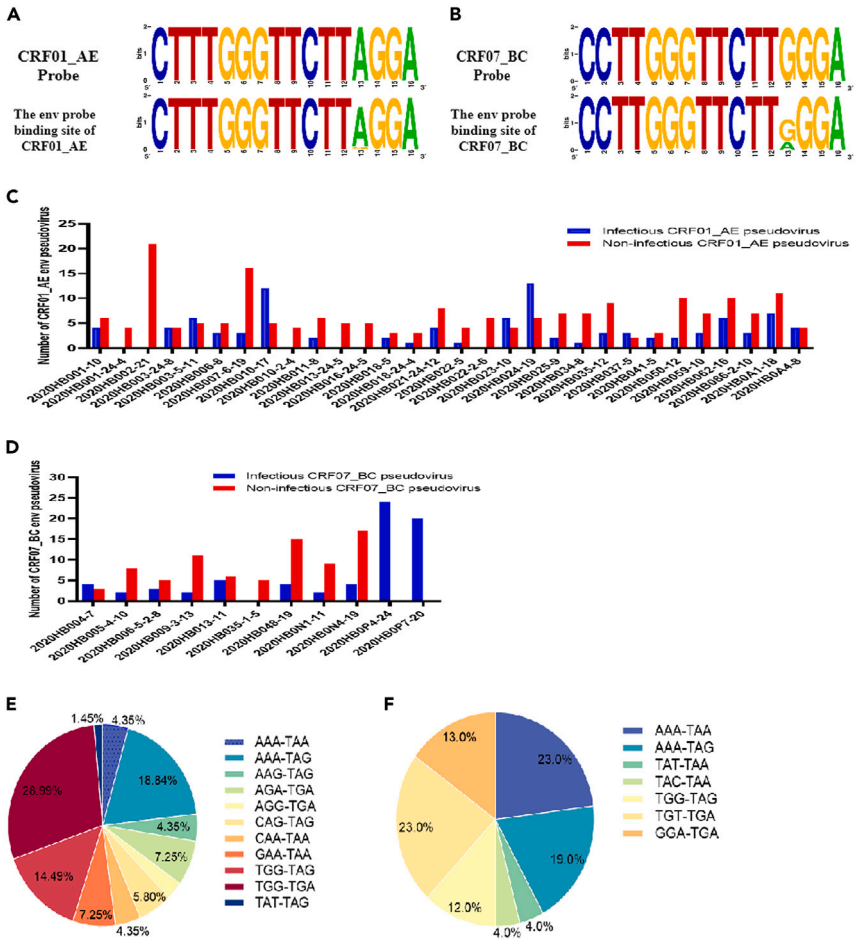

Figure 5. The effect of mutations in env gene outside the probe binding site on the IPDA

The frequency of each amino acid in the sequences isolated from all patients infected with CRF01_AE (n = 294) (A) or CRF07_BC (n = 149) (B) was analyzed. The infectivity of CRF01_AE (C) and CRF07_BC (D) pseudovirus was detected using TZM-bl cells. The mutation characteristics of env gene of CRF01_AE (E) or CRF07_BC (F) outside the probe binding site were analyzed.

See also Figure S1.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.109941.

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AUTHOR CONTRIBUTIONS

Z. Zhou, X.Z., M.W., F.J., and J.T. conducted the experimental procedures, contributed research results, and worked with W.H. and P.S. to design the project. Z. Zhou wrote the main content of the article, and H.Z. and W.H. provided edits. All authors have reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coliDH5a	Takara	Cat#9057
Biological samples		
Peripheral blood (HIV-1 positive)	Baoding Medical College	N/A
Chemicals, peptides, and recombinant proteins		
DMEM Medium	Hyclone	Cat#SH30243.01B
0.25%trypsin-EDTA	Gibco	Cat#25200
PBS	Hyclone	Cat#SH30256.01B
Pen/Strep	Gino Bio Co.	Cat#GNM15140
FBS	PAN-Seratech	Cat#ST30-2602
Bright-GloTM	PerkinElmer	Cat#6066769
DMSO	SIGMA	Cat#D2650-100ML
Peptone	OXOID	Cat#500Grams/LP0042
Yeast	OXOID	Cat#500Grams/LP0021
Lipofectamine 3000	Invitrogen	Cat#L3000015
Opti-MEM Reduced Serum Medium	Gibco	Cat#31985088
NaCl	Sinopharm	Cat#10019308
PerfeCTa qPCR ToughMix UNG, 2×	Quantabio	Cat#95138
Sapphire Chip	Stilla Technologies	Cat#C14012
Experimental models: Cell lines		
Tzm-bl cell line	BioVector NTCC Inc.	Cat#3617197
Critical commercial assays		
Plasmid miniaturization kit	Zoman	Cat#ZP101-3
Plasmid extraction kit	Qiagen	Cat#12965
Oligonucleotides		
HIV-1 primers	This study	Table S1
Software and algorithms		
Naica™ Crystal Digital PCR System	Stilla Technologies	Naica
Prism 8	GraphPad Software	https://www.graphpad.com/

RESOURCES AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Weijin Huang (huangweijin@nifdc.org.cn).

Materials availablility

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human subject samples

We recruited HIV-1-infected individuals at the People's Hospital of Baoding in Hebei, China, and obtained their informed consent. Ethical approval to conduct this study was obtained from the People's Hospital of Baoding. Peripheral blood mononuclear cells (PBMCs) from blood samples were collected from HIV-1-infected individuals. Blood was collected at the time of ART initiation and 6-monthly intervals.

ddPCR system

The naica® system is an easy-to-use dPCR platform that harnesses cutting-edge microfluidic technology to integrate the dPCR workflow onto a single consumable chip. The technology, known as Crystal Digital PCR™, partitions samples into a large array of thousands of individual droplet crystals – each its own reaction compartment – before amplifying nucleic acid molecules in each droplet crystal. These reactions are tagged with fluorophores to be read using up to six different fluorescence light channels. This makes for a fast and simple workflow that provides high multiplexing capability with powerful sensitivity while reducing time to results and saving precious sample.

METHOD DETAILS

DNA extraction, amplification and sequencing

DNA was extracted from PBMCs using the Qiagen DNA Mini Kit (Qiagen, Germany). The *env* of the HIV-1 genome was amplified by nested polymerase chain reaction (PCR) using three primers targeting different HIV-1 subtypes (Table S1). The conditions were as follows: the first-round PCR was carried out at 94°C for 4 minutes, followed by 30 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 68°C for 4 minutes, with a final extension at 68°C for 10 minutes. The second-round PCR was carried out at 94°C for 4 minutes, followed by 30 cycles of 94°C for 15 seconds, 60°C for 30 seconds and 68°C for 3 minutes, with a final extension at 68°C for 10 minutes. The PCR products were identified by agarose gel electrophoresis, and the positive amplicons were sent to Nuosa Biomedicine Co. for sequencing.

Construction of pseudoviruses

Full-length *env* PCR products were cloned and inserted into pCDNA3.1/V5-His-TOPO. Pseudoviruses were prepared by transfecting 293T cells with the HIV-1 *env* clone and the *env*-deficient HIV-1 backbone plasmid pSG3Δenv using Lipofectamine 3000 reagent (Invitrogen), and the supernatant was harvested for purification 48 hours after transfection. TZM-bl cells were infected with purified pseudovirus, and the RLU value was measured at 48 hours. If the RLU value was more than 10 times higher than that of the control, the pseudovirus was considered to be infective. The higher the RLU value is, the higher the infectivity.

Phylogenetic analyses

We extracted DNA from PBMC samples of HIV-1 positive individual, and env sequences were amplified and sequenced. These env sequences were aligned with HIV-1 reference strains using Cluster X2. Maximum-likelihood trees of env sequences were constructed using Mega-X software, and a general time-reversible (GTR) nucleotide substitution model with a gamma distribution of rates based on 1000 bootstrap replicates was employed.

Intact proviral DNA assay (IPDA)

DNA was extracted from 5×106 PBMCs using the Qiagen DNA Mini Kit (Qiagen, Germany). DNA concentrations were determined using a Qubit3.0 instrument. Quantification of intact HIV-1 was carried out by optimized primers/probes for different HIV-1 subtypes. The Ψ amplicon is positioned at HXB2 coordinates 692–797, and the RRE of the proviral genome is positioned at HXB2 coordinates 7736–7851. The host gene RPP30 was used to provide a measure of input cell number. The reaction mix comprised primers/probes, master mix, fluorescein, DNA and water. Then, four 25 μL aliquots of the PCR mixture were loaded onto a sapphire chip, and up to three chips were placed in a Naica Geode system for division into 20,000 to 30,000 droplets for PCR. The PCR conditions were the same as those for the RT–qPCR system. Then, the sapphire chip was transferred to a Prism3 fluorescence reader for imaging.

QUANTIFICATION AND STATISTICAL ANALYSIS

The rest of numerical data were analyzed with GraphPad Prism version 8 using statistical test noted within the corresponding figure captions.