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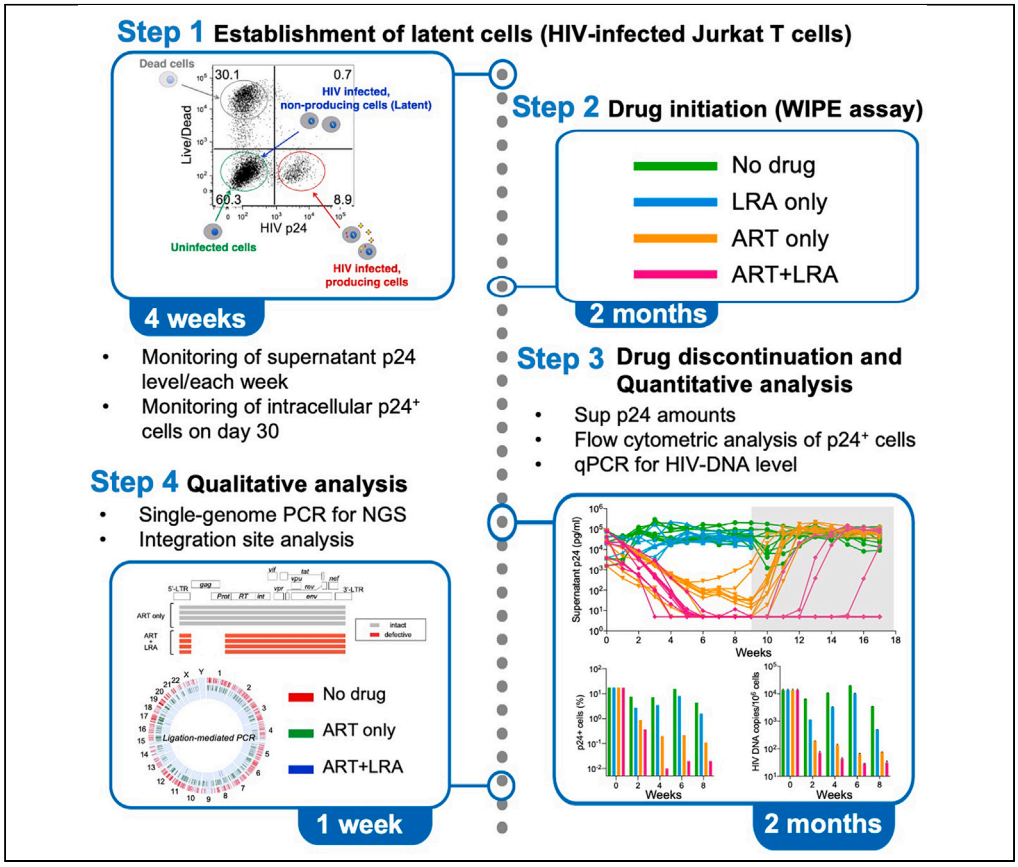
Assessing the effects of antiretroviral therapy-latency-reversing agent combination therapy on eradicating replication-competent HIV provirus in a Jurkat cell culture model



Eradication of HIV-1 latently infected cells is an important issue in HIV treatment. However, there are limited models available to assess therapeutic efficacy *in vitro*. Here, we present a protocol for establishing a variety of HIV-infected Jurkat cells, including productive and latent status, evaluating the efficacy of antiviral agents, followed by PCR/sequencing-based detection of replication competent HIV provirus. This protocol is useful for optimization of treatment of HIV-1 and provides insights into the mechanisms of clonal selection of heterogeneous HIV-1-infected cells.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for the widely distributed HIV-1 provirus elimination (WIPE) assay

Establishment of HIV-1 latently infected Jurkat cells for long-term culture

Assessment of viral re-emergence upon LRA and ART combination therapy

Detection of replication-competent HIV provirus and integration sites by PCR and sequencing

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Assessing the effects of antiretroviral therapy-latency-reversing agent combination therapy on eradicating replication-competent HIV provirus in a Jurkat cell culture model

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SUMMARY

Eradication of HIV-1 latently infected cells is an important issue in HIV treatment. However, there are limited models available to assess therapeutic efficacy *in vitro*. Here, we present a protocol for establishing a variety of HIV-infected Jurkat cells, including productive and latent status, evaluating the efficacy of antiviral agents, followed by PCR/sequencing-based detection of replication competent HIV provirus. This protocol is useful for optimization of treatment of HIV-1 and provides insights into the mechanisms of clonal selection of heterogeneous HIV-1-infected cells.

For complete details on the use and execution of this protocol, please refer to Matsuda et al. (2021).[1]

BEFORE YOU BEGIN

This protocol was used in a recent publication to establish a long-term cell culture system that harbors thousands of different HIV-1infected cell clones with a wide distribution of HIV-1 provirus, similar to that observed *in vivo*.[1] We used the system to evaluate whether the addition of latency-reversing agents (LRAs) to antiretroviral therapy (ART) contributes to a reduction in/elimination of HIV-latently infected cells. We recommend a thorough follow-up of this protocol, which may take several months (at least 5 months) to complete. Because this protocol is specifically designed with high containment viruses used in a Biosafety Level 3 (BSL3) laboratories in mind, it requires standard laboratory equipment, including a BSL3 laboratory safety cabinet. Infectious samples require inactivation with the lysis buffer in the case of culture supernatants and in the case of infected cells after fixation. In addition, there is the necessary equipment to perform the assay, including a flow cytometer, sonication device, real-time PCR machine, and next generation sequence (NGS) system to evaluate details of the HIV-1 provirus.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-HIV-1 p24-PE mAb (KC57)	Beckman Coulter (Brea, CA)	6604667
Anti-HIV-1 p24-APC mAb (28B7)	MediMabs (Montréal, Canada)	MM-0289-APC

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
HIV-1 NL4-3 Infectious Molecular Clone (pNL-43)	NIH AIDS Reagent Program	ARP-114
HIV-1 JR-FL Infectious Molecular Clone (pJR-FL)	Kindly provided by Dr. Yoshio Koyanagi	N/A
Chemicals, peptides, and recombinant proteins		
RPMI 1640	Sigma-Aldrich (St. Louis, MO)	R8758
Fetal calf serum (FCS)	Sigma-Aldrich (St. Louis, MO)	172012
Triton X-100	Sigma-Aldrich (St. Louis, MO)	T9284
Phosphate-buffered salts (PBS)	Wako Pure Chemical (Osaka, Japan)	045-29795
4% Paraformaldehyde phosphate-buffered solution	Wako Pure Chemical (Osaka, Japan)	163-20145
Flow cytometry perm buffer	TONBO Biosciences (San Diego, CA)	TNB-1213-L150
Ghost Dye Red 780	TONBO Biosciences (San Diego, CA)	13-0865
PEP005	Cayman Chemical (Ann Arbor, MI)	16207
EFdA/MK-8591/ISL	Nakata et al., 2007[13]	N/A
TAF	Sigma-Aldrich (St. Louis, MO)	SML3219
AZT	Tokyo Chemical Industry (Tokyo, Japan)	A2052
FTC	Tokyo Chemical Industry (Tokyo, Japan)	E1007
3TC	Tokyo Chemical Industry (Tokyo, Japan)	L0217
Phorbole 12-myristate13-acetate/ PMA	Wako Pure Chemical (Osaka, Japan)	162-23591
Recombinant Human TNF-α	BioLegend (San Diego, CA)	570106
Critical commercial assays		
Lumipulse HIV Ag/Ab kit	Fujirebio (Tokyo, Japan)	295335
HIV Type 1 p24 Antigen ELISA kit	ZeptoMetrix Corp (Buffalo, NY)	0801111
Flow Cytometry Perm Buffer	TONBO Biosciences (San Diego)	TNB-1213
QIAmp DNA Blood mini kit	Qiagen (Hilden, Germany)	51104
RNeasy Mini Kit	Qiagen (Hilden, Germany)	74004
Premix Ex Taq (Probe qPCR) ROX plus	Takara Bio (Shiga, Japan)	RR39LR
PrimeScript RT Master Mix	Takara Bio (Shiga, Japan)	RR036A
Takara Ex Taq hot start version	Takara Bio (Shiga, Japan)	R007A
PowerUp SYBR Green Master Mix	Applied Biosystems	A25742
QIAquick PCR Purification Kit	Qiagen (Hilden, Germany)	28104
NEBNext Ultra II DNA Library Prep Kit for Illumina	New England Biolabs (Ipswich, MA)	E7645S
Qubit dsDNA High Sensitivity Assay kit	Invitrogen (Waltham, MA)	Q32851
Library quality check by TapeStation	Agilent Technologies (Santa Clara, CA)	https://www.agilent.com/en/promotions/agilent-2200-tapestation-system
GenNext NGS Library Quantification kit	TOYOBO (Osaka, Japan)	NLQ-101
NEBNext Ultra II End Repair/dA-Tailing Module	New England Biolabs (Ipswich, MA)	E7546S
NEB Next Ultra II Ligation Module	New England Biolabs (Ipswich, MA)	E7595S
AMPure XP Beads	Beckman Coulter (Brea, CA)	A63880
Q5 Hot Start High-Fidelity DNA Polymerase	New England Biolabs (Ipswich, MA)	M0493S
ddPCR Supermix for Probes	Bio-Rad (Hercules, CA)	186-3026
Deposited data		
Fastq of all samples	This study	DRA accession number: DRA012587 DRA012818
Experimental models: cell lines		
Human: Jurkat cells	ATCC	TIB-152
Oligonucleotides		
HIV-1 LTR forward primer for qPCR: 5′-TGT GTGCCCGTCTGTTGTGT-3′	Butler et al.[2]	N/A
HIV-1 LTR reverse primer for qPCR: 5′-GAG TCCTGCGTCGAGAGAGC-3′	Butler et al.[2]	N/A
HIV-1 LTR probe for qPCR: 5′-FAM-CAGT GGCGCCCGAACAGGGA-BHQ1-3′	Butler et al.[2]	N/A

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
β2m forward primer for qPCR: 5′-GGAATTG ATTTGGGAGAGCATC-3′	Goff et al.[3]	N/A
β2m reverse primer for qPCR: 5′-CAGGTC CTGGCTCTACAATTTACTAA-3′	Goff et al.[3]	N/A
β2m probe for qPCR: 5′-FAM-AGTGTGAC TGGGCAGATCATCCACCTTC-BHQ1-3′	Goff et al.[3]	N/A
HIV-1 gag forward primer for qPCR and ddPCR: 5′-GGTGCGAGAGCGTCGGTATTAAG-3′	Douek et al.[4]	N/A
HIV-1 gag reverse primer for qPCR and ddPCR: 5′-AGCTCCCTGCTTGCCCATA-3′	Douek et al.[4]	N/A
β-actin forward primer for qPCR: 5′-GCGAG AAGATGACCCAGATC-3′	Hattori et al.[5]	N/A
β-actin reverse primer for qPCR: 5′-CCAGT GGTACGGCCAGAGG-3′	Hattori et al.[5]	N/A
HIV-1 gag probe for ddPCR: 5′-/HEX/ AAAATTCGG/ ZEN/ TTAAGGCCAGGGGGAAAGAA/3IABkFQ/-3′	Iwase et al.[6]	N/A
Albumin forward primer for ddPCR: 5′-TGCATG AGAAAACGCCAGTAA-3′	Douek et al.[4]	N/A
Albumin reverse primer for ddPCR: 5′-ATGGTC GCCTGTTCACCAA-3′	Douek et al.[4]	N/A
Albumin probe for ddPCR: 5′-6-FAM/TGA CAG AGT/ZEN/CACCAAATGCTGCACAGAA/ 3IABkFQ/ -3′	Iwase et al.[6]	N/A
Forward primer for 1st near full-length single HIV-1 genome PCR: 5′-AAATCTCTA GCAGTGGCGCCCGAACAG-3′	Imamichi et al.[7]	N/A
Reverse primer for 1st near full-length single HIV-1 genome PCR: 5′-TGAGGGATC TCTAGTTACCAGAGTC-3′	Imamichi et al.[7]	N/A
Forward primer for 2nd near full-length single HIV-1 genome PCR: 5′-GCGCCCGA ACAGGGACYTGAAARCGAAAG-3′	Imamichi et al.[7]	N/A
Reverse primer for 2nd near full-length single HIV-1 genome PCR: 5′-GCACTCAAG GCAAGCTTTATTGAGGCTTA-3′	Imamichi et al.[7]	N/A
Long linker for LM PCR: 5′-TCATATAATGGG ACGATCACAAGCAGAAGACGGCATACGAG ATNNNNNNNN CGGTCTCGGCATTCCTGC TGAACCGCTCTTCCGATCT-3′	Satou et al.[8]	N/A
Short linker for LM PCR: 5′-p-GATCGGAAGA GCGAAAAAAAAAAAAA-3′	Satou et al.[8]	N/A
Primer (B3) for 1st LM-PCR: 5′-GCTTGCCTT GAGTGCTTCAAGTAGTGTG-3′	Satou et al.[8]	N/A
Primer (B4) for 1st LM-PCR: 5′- TCATGATCA ATGGGACGATCA-3′	Satou et al.[8]	N/A
Primer (P5B5) for 2nd LM-PCR: 5′-AATGATAC GGCGACCACCGAGATCTACACGTGCCCGT CTGTTGTGTGACTCTGG-3′	Satou et al.[8]	N/A
Primer (P7) for 2nd LM-PCR: 5′-CAAGCAGAA GACGGCATACGAGAT-3′	Satou et al.[8]	N/A
Sequencing primer for LM-PCR (‘Read1’ targeting HIV-1): 5′-ATCCCTCAGACCCTTTT AGTCAGTGTGGAAAATCTC-3′	Satou et al.[8]	N/A
Sequencing primer for LM-PCR (‘Read2’ targeting human genome): 5-CGGTCTCGGC ATTCCTGCTGAACCGCTCTTCCGATCT-3′	Satou et al.[8]	N/A
Sequencing primer for LM-PCR (‘Index1’ targeting adapter barcode): 5-GATCGGAAGA GCGGTTCAGCAGGAATGCCGAGACCG-3′	Satou et al.[8]	N/A

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
FlowJo	Tree Star (Ashland, OR)	https://www.flowjo.com
Prism 8	GraphPad Software (San Diego, CA)	https://www.graphpad.com/
BWA-MEM algorithm	Li et al.[9]	http://bio-bwa.sourceforge.net/
Samtools	Li et al.[9]	http://samtools.sourceforge.net/
Picard	Broad Institute of MIT and Harvard	http://broadinstitute.github.io/picard/
Integrative Genomics Viewer	Robinson et al.[10]	http://software.broadinstitute.org/ software/igv/

MATERIALS AND EQUIPMENT

FACS buffer		
Reagent	Final concentration	Amount
PBS	N/A	489.5 mL
Fetal bovine serum (FBS)	2.0%	10 mL
NaN_3	0.1%	0.5 mL
Total	N/A	500 mL

Note: Storage conditions: 4°C, maximum storage time for 6 months.

Lysis buffer		
Reagent	Final concentration	Amount
Triton X-100	1.0%	5.0 mL
ddH_2O	N/A	495.0 mL
Total	N/A	500 mL

Note: Storage conditions: 20°C–25°C, maximum storage time for 6 months to 1 year.

Intracellular antibody master mix	
Antibody	Dilution factor
Anti-HIV-1 p24-PE mAb (KC57)	1:100
Anti-HIV-1 p24-APC mAb (28B7)	1:100
Diluent	FACS buffer
Total volume (per sample)	10 μL

Note: Storage conditions: 4°C, maximum storage time for 6 months.

STEP-BY-STEP METHOD DETAILS

Establishment of latent cells (HIV-infected non-producing cells)

Timing: 4 weeks (for all steps in this section)

Timing: 3 days (for steps 1 to 2)

Timing: 1 h (for step 3)

Timing: 30 days (for steps 4 to 5)

Timing: 2 h (for step 6)

For this protocol, we will describe the establishment of HIV-1 latently infected cells that harbor widely infected clones similar to that observed *in vivo*.

1. Preparation of cells.
   a. Obtain a human T cell-derived cell line, Jurkat cells to establish cell populations chronically infected with HIV-1.
   b. Maintain in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (Sigma-Aldrich), 50 U/mL penicillin and 50 μg/mL streptomycin, in 25 cm^2 flask.
   c. Culture in a 5% CO2 humidified incubator at 37°C.
   d. When cells become 70%–80% confluent (maximum cell numbers 2 × 10^6 cells/mL), split to 1/10 and passage.
2. Virus preparation and determination of virus titer.
   a. Obtain a human embryonic kidney cell line, HEK293T cells and maintain in DMEM medium (Sigma-Aldrich) supplemented with 10% fetal calf serum, 50 U/mL penicillin and 50 μg/mL streptomycin, in 10 cm dish.
   b. Transfect the proviral plasmid HIV-1_{NL4-3} or HIV-1_{JR-FL} to HEK293T cells to produce virus.
   c. Collect the culture supernatant and determine the p24 concentration using a p24 antigen ELISA kit (ZeptoMetrix Corp., Buffalo, NY) and store at 80°C until use.
3. HIV-1 infection.
   a. Infect 1 × 10^6 Jurkat cells with the HIV-1 wild type (HIV-1_{NL4-3} or HIV-1_{JRFL}) at 50 ng of p24/mL in a 10 mL culture condition in a 25 cm^2 flask. The cell density should not exceed 5 × 10^6 cells/mL.
   b. Culture without wash until cells are confluent.
4. Maintain HIV-1 infected cells.
   a. Culture the infected cells in 10 mL of RPMI 1640 medium supplemented with 10% fetal calf serum, 50 U/mL penicillin and 50 μg/mL streptomycin, in 25 cm^2 flask.
   b. Passage weekly in 1/10 splits at confluence (so that cell numbers are less than 5 × 10^6 cells/mL).
5. Weekly monitoring of p24 level in the supernatant.
   a. Measure HIV-1 p24 levels in the cell culture supernatant using commercially available p24 ELISA kit or Lumipulse system (FUJIREBIO).

   Note: When using the Lumipulse system, the infected supernatant should be diluted 10- to 100-fold with lysis buffer and require inactivation treatment prior to measurement.

6. Monitor the number of intracellular $p24^+$ cells on day 30 after HIV-1 infection by flow cytometry.

   Note: Use our latest flow cytometric method of intracellular staining described by our group[11], which is a modification of our previously published method.

   ⚠ CRITICAL: The proportion of intracellular p24-positive cells in the cell cultures should be around 2.5%–5% of the total number of cells. THIS STEP CAN CRITICALLY AFFECT THE RESULTS. Indeed, cultures of Jurkat cells containing intracellular $p24^+$ cells of 2.5% or 15% (mixtures of HIV-infected and -uninfected Jurkat cells), showed differences in the rate of virus elimination after long-term culture as well differences in rebound after treatment cessation (Figures 1A and 1B). We recommend determination of the percentage of reactivation-competent HIV-latently-infected cells by HIV flow cytometry. This allowed us to determine the percentage of latent cells that can be reactivated by TNF-alpha (see[12] for details).

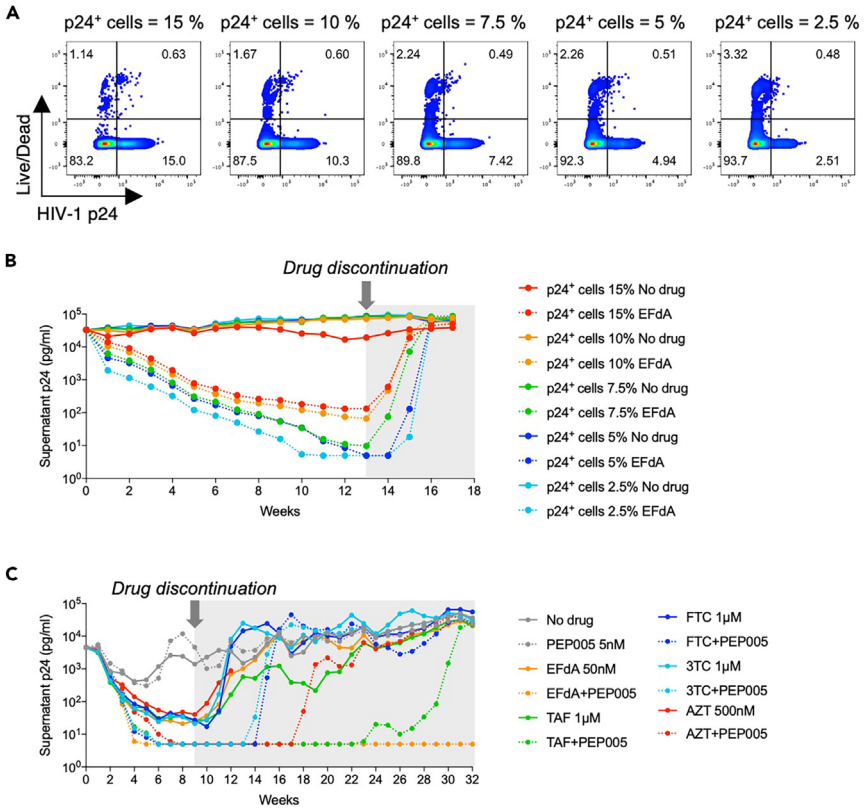

Figure 1. Culture protocol of intracellular p24-positive cells and selection of antiviral agents for WIPE assay

(A) HIV-infected cells with different proportions of intracellular p24-positive cells at the initial culture period. Note the different proportion of intracellular p24-positive cells (from 2.5% to 15%) representing a mixture of HIV-infected and uninfected Jurkat cells (see Graphical Abstract for the meaning of the numbers in different quadrants of the graphs).

(B) Changes in supernatant p24 levels in the absence (line) and presence of 50 nM EFdA (dotted line). EFdA treatment was terminated on week 13, but analysis continued for additional 4 weeks. EFdA affected the rate of virus reduction during the long-term culture and rebound rate after drug discontinuation.

(C) Comparison of the effects of different NRTIs and their combinations with an LRA, 5nM PEP005, on supernatant p24 levels. Drug treatment was terminated on week 9, but analysis continued for additional 23 weeks.

Determination of antiviral activity of LRAs and conventional anti-HIV-1 agents in HIV-infected Jurkat cells (WIPE assay)

Timing: 4 months (for all steps in this section)

Timing: 2 h (for steps 7a to 7b)

Timing: 9 weeks (for steps 7c to 7g)

Timing: 8 weeks (for step 8)

Timing: 2 days (for step 9)

Next, we evaluated the efficacy of the combinations of ART and LRAs against HIV-1-latently infected Jurkat cells using an *in vitro* culture system. The aim of this assay is to identify the most efficacious ART-LRA combination therapy. We reported recently that the combination of nucleoside reverse transcriptase inhibitors (NRTIs), islatravir (EFdA, MK-8591), plus a PKC activator, PEP005, potently eradicated HIV-1 latently infected cells *in vitro*.

7. Prepare HIV-infected Jurkat cell cultures:
   a. Seed 5.0 × 10^4 cells/mL of HIV-infected Jurkat onto 12-well plate (2 mL each well).
   b. Add a single ART (e.g., NRTI, integrase inhibitor, or protease inhibitor), a single LRA (e.g., PKC activator, HDAC inhibitor, BET inhibitor) or a combination of the two types of agents.
   c. Passage weekly to maintain cell numbers <5 × 10^6 cells/mL when confluent.
   d. Use different doses of the test drugs.
   e. Monitor the supernatant p24 level once every week.
      i. Measure HIV-1 p24 levels in the cell culture supernatant as described in Step5a.
   f. Monitor intracellular HIV DNA copies.
      i. Measure proviral DNA copies by quantitative PCR (qPCR) analysis.
   g. Monitor reactivation-competent HIV-latently infected cells.
      i. Collect the cells, wash twice with PBS, and seed into 24-well plates at 5.0 × 10^5 cells/mL.
      ii. Treat the cells with 10 ng/mL TNF-alpha for 24 h.
      iii. Detect intracellular p24-double positive cells (see HIV Flow methods: Pardons et al.[12]). Subtract the percentage of unstimulated p24-positive cells from p24-positive cells after TNF-alpha stimulation to calculate the percentage of reactivation-competent HIV latently infected cells.

Note: Use our latest qPCR method described by our previously published method.[1] For more detail, please see the Note below.

Note: Quantitative PCR (qPCR) analysis (step 7f)

qPCR for intracellular HIV-1 DNA levels should be conduct using Premix Ex Taq (Probe qPCR) Rox plus (Takara Bio). The oligonucleotides HIV-1 LTR and β2-microgloblin (β2m) are used for HIV-1 DNA quantitation and cell number determination, respectively (see key resources table). We recommend calculating the HIV proviral DNA copy and cell numbers based on a standard curve generated using a serially diluted HIV-1 pNL4-3 plasmid and DNA extracted from Jurkat cells, respectively.

8. Cessation of treatment:
   a. Approximately 9 weeks of treatment with a single agent or combination of agents should reduce the viral load in the supernatant. Then, stop the drug treatment.
   b. Maintain cultivation for another 8 weeks without drug treatment.
   c. Monitor the p24 level in the supernatant and measure intracellular HIV DNA level weekly during cell culture.
9. Stimulation:
   a. At the end of the treatment-cessation period (8 weeks), stimulate the previously drug-treated cells with 10 ng/mL TNF alpha and determine viral recurrence as described Step7 g.
   b. Confirm the rise in HIV-1 mRNA expression by qPCR or intracellular p24 staining.

⚠ CRITICAL: It is desirable to keep the viral load in the supernatant below the detection limit upon cessation of drug treatment. Since it is preferable for the viral load in the culture supernatant to fall below the detection limit during the drug treatment period before the treatment cessation step, the treatment period can be further extended from 9 weeks if the viral load does not fall below the detection limit. Generally, the viral load is reduced to near the detection limit with ART alone, but it may rebound during the treatment period due to drug resistance mutations or other problems. On the other hand, when ART is combined with a drug that activates directly on latently infected cells, such as LRA, the amount of virus in the culture supernatant often declines below the detection limit within the 9-week treatment period.

Near-full-length single HIV-1 genome PCR

Timing: 2–3 days (for all steps in this section)

Table 1. PCR mixture for ddPCR

Component	Concentration	Amount
ddPCR Supermix for Probes (Bio-Rad, Hercules, CA).	2×	11 μL
Albumin (Alb) probe	10 μM	0.55 μL
Gag probe	10 μM	0.55 μL
Albumin primers (Forward + Reverse)	10 μM	1.32 μL
Gag primers (Forward + Reverse)	10 μM	1.76 μL
PCR grade water		Variable (Depends on the Input DNA amount)
DNA		Up to 200 ng (Or the maximum volume permissible)
Total volume		22 μL

Timing: 1–2 h (for step 10)

Timing: 4–5 h (for step 11)

Timing: 1 day (for steps 12 to 14)

We next performed near-full length HIV-1 genome PCR to determine whether the combination of ART and LRAs eradicated intact or replication-competent HIV provirus. The PCR protocol used in this step was described previously[7] with some modifications (see key resources table and Table 1 for primers and PCR conditions, respectively).

10. DNA extraction.
    a. Extract DNA from the cells using QIAamp DNA Blood Mini Kit (QIAGEN) following the instructions provided by the manufacturer.
    b. Resuspend the DNA in 100 μL nuclease-free water.
11. Execute digital droplet PCR (ddPCR).
    a. Prepare ddPCR reaction mix with the following composition provided in Table 1.
    b. Generate droplets using the QX200 droplet generator (Bio-Rad) following manufacturer's instructions (QX200™ Droplet generator Instruction manual, catalog#186–4002).
    c. Once droplet generation is completed, transfer the droplets into a 96-well PCR plate and seal with a preheated PX1 PCR plate sealer (Bio-Rad).
    d. Incubate in a thermal cycler under the conditions summarized in Table 2.
    e. After PCR reaction, place the plate in the QX200 droplet reader (Bio-Rad) and quantify both the positive and negative droplets based on fluorescence.
    f. Determine the threshold value based on the highest value of droplet fluorescence in the no template control to provide an objective cut-off value (Figure 2A).
    g. Calculate the copy number of target genes (*alb* and *gag*) using QuantaSoft v1.7.4 (Bio-Rad).
    h. Calculate the proviral load as follows: Proviral load (per cell) = (*gag* copy number) / [(*alb* copy number) / 2]
12. Single genome PCR (first-round PCR).
    a. Amplify the single genome with 1 copy/tube of DNAs using Takara Ex Taq hot start version (TaKaRa Bio, Shiga, Japan). Calculate the input DNA amount (ng) using the following formula.:

Table 2. PCR conditions for ddPCR

Temperature	Time	Cycles
95°C	10 min	1
94°C	30 s	39
58°C	1 min	
98°C	10 min	1
4°C	Hold	Hold

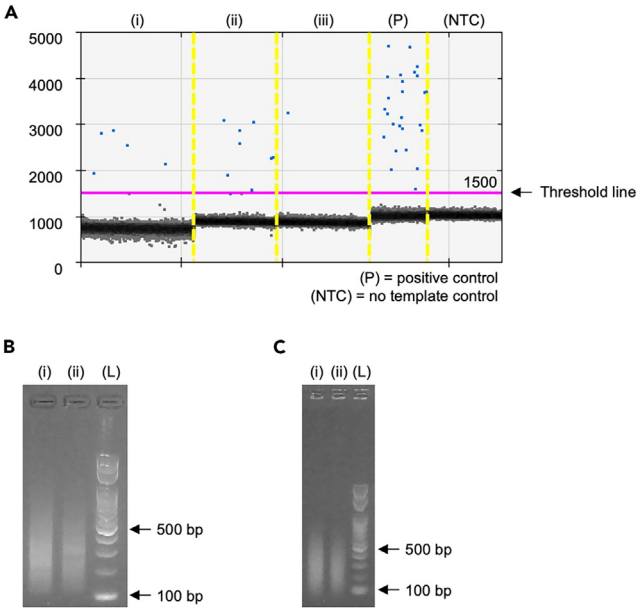

Figure 2. Representative results of ddPCR and LM-PCR

(A) Representative ddPCR result for *gag* gene. Columns (i) – (iii) are tested samples while column (P) is a positive control (in this case, DNA from Jurkat cells spiked with ACH-2 cells). The threshold line (purple) is determined based on the highest droplet fluorescence in the NTC (no template control) column.

(B) Representative gel image of post-sonication DNA. Note the smear from 100 – 1000 bp.

(C) Representative gel image of LM-PCR library after the 2nd nested PCR. Note the smear from 100 – 500 bp.

$$Required\ amount\ of\ DNA\ (ng) = \frac{Input\ DNA\ amount\ (ng)\ in\ ddPCR}{gag\ copy\ number\ in\ generated\ droplets}$$

Detail PCR mixture is given in Table 3.

b. Refer to the table below for PCR conditions (Table 4).

13. Single genome PCR (second-round PCR).
    a. Dilute the first-round PCR products 1:50 in PCR-grade water and use 5 μL of the diluted mixture for second-round amplification. Detail 2nd PCR mixture information is given in Table 5.
    b. Refer to the table below for PCR conditions (Table 6).
14. Electrophoresis to confirm single genome.
    a. Prepare 1% agarose gel and perform electrophoresis.
    b. Based on Poisson distribution, samples with ≤30% positive reactions are considered to contain a single HIV-1 genome and are selected for sequencing.

NGS analysis of near-full length HIV-1 genome

Timing: 2–3 days (for all steps in this section)

Timing: 2–3 h (for step 15)

Timing: 1–2 days (for step 16)

We next performed next-generation sequencing (NGS) analysis of the near-full length HIV-1 genome.

Table 3. PCR mixture for NFL-single genome PCR (First round PCR)

Component	Concentration	Amount
Ex Taq HS	5 U/μL	0.125 μL
Ex Taq buffer (EX Taq)	10×	2.5 μL
dNTP mixture	2.5 mM	2 μL
Forward primer for 1st near full-length single HIV-1	10 μM	1.75 μL
Reverse primer for 1st near full-length single HIV-1	10 μM	1.75 μL
PCR grade water		Variable (Depends on the Input DNA amount)
DNA		Variable (Depends on the calculation performed in 12(a)
Total volume		25 μL

15. DNA purification and fragmentation for NGS.
    a. Purify the amplified PCR products of the selected samples using the QIAquick PCR Purification Kit (Qiagen) and follow the protocol recommended by the manufacturer.
    b. Determine the concentration of PCR purified products using Qubit Fluorometer.
    c. Take 2 μg PCR purified DNA and add TE buffer to make up the volume up to 100 μL.

Note: To achieve an average fragment size of 300–400 bp, begin with 2 μg of PCR purified DNA and supplement it with TE buffer to reach a final volume of 100 μL.

Note: Employ a Picoruptor device from Diagenode (Liege, Belgium) for sonication, utilizing the following program at a temperature of 4°C: 30 s of sonication followed by 90 s of rest, repeated for a total of 6 cycles.

16. Preparation of libraries for NGS.
    a. Prepare the libraries using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs) according to the instructions supplied by manufacturer.

Ligation-mediated PCR

Timing: 2–3 days (for all steps in this section)

Timing: 1–2 h (for step 17a)

Timing: 1–2 days (for steps 17b to 21)

Table 4. PCR conditions for first-round PCR

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1
Denaturation	95°C	10 s	5
Annealing	66°C	10 s	
Extension	68°C	7 min	
Denaturation	95°C	10 s	5
Annealing	63°C	10 s	
Extension	68°C	7 min	
Denaturation	95°C	10 s	5
Annealing	61°C	10 s	
Extension	68°C	7 min	
Denaturation	95°C	10 s	15
Annealing	58°C	10 s	
Extension	68°C	7 min	
Final extension	68°C	5 min	1
Hold	4°C	forever	

Table 5. PCR mixture for NFL-single genome PCR (Second round PCR)

Component	Concentration	Amount
Ex Taq HS	5 U/μL	0.125 μL
Ex Taq buffer (EX Taq)	10×	2.5 μL
dNTP mixture	2.5 mM	2 μL
Forward primer for 2nd near full-length single HIV-1	10 μM	1.75 μL
Reverse primer for 2nd near full-length single HIV-1	10 μM	1.75 μL
PCR grade water		Variable (Depends on the Input DNA amount)
DNA		Variable (Depends on the calculation performed in 12(a)
Total volume		25 μL

Timing: 1–2 days (for step 22)

The next step included ligation-mediated PCR to determine the sites of integration of HIV-1 provirus DNA using the protocol described previously[8] with minor modifications (see key resources table and Table 9; Table 11 for PCR primers and conditions, respectively).

17. DNA fragmentation.
    a. Prepare up to 2 μg DNA in Tris-EDTA buffer (total volume up to 100 μL).
    b. Shear the DNA by sonication using a Picoruptor device (Diagenode, Liege, Belgium) to obtain fragments with an average size of 300–400 bp utilizing the following program at a temperature of 4°C: 30 s of sonication followed by 90 s of rest, repeated for a total of 6 cycles.
    c. Load with run 5 μL of sonication product on 2% agarose gel to confirm obtaining the appropriate fragment sizes (Figure 2B).
18. End-repair and adapter ligation:
    a. Use 50 μL of the sonication product for end-repair using the NEB Next Ultra II End Repair/dA-tailing Module and follow the protocol provided by the manufacturer.
    b. Once reaction is completed, proceed immediately to adapter ligation using the NEB Next Ultra II Ligation module, as described in the protocol provided by the manufacturer.
    c. Refer to the Table below for incubation conditions (Table 7).
    d. Purify the post-adapter ligated DNA using the QIAquick PCR Purification Kit (Qiagen) according to the instructions supplied by the manufacturer and elute the DNA in 50 μL ddH_2O.
19. Size selection.
    a. Perform size selection using 0.8× volume (40 μL) of AMPure XP Beads based on the instructions provided by the manufacturer.
    b. For the final step, elute with 65 μL ddH_2O and transfer the supernatant to a new, clean tube.
20. First external PCR.
    a. Amplify the post-size selection product using the Q5 Hot Start High Fidelity DNA Polymerase and the primer pairs B3 and B4. Refer to the table below for PCR mixture recipe (Table 8).

Table 6. PCR conditions for second-round PCR

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	2 min	1
Denaturation	95°C	10 s	8
Annealing	68°C	10 s	
Extension	68°C	7 min	
Denaturation	95°C	10 s	12
Annealing	65°C	10 s	
Extension	68°C	7 min	
Final extension	68°C	5 min	1
Hold	4°C	forever	

Table 7. Incubation conditions for End-repair and adapter ligation

Temperature	Time
End Repair	
20°C	30 min
65°C	30 min
Adapter Ligation	
20°C	15 min
37°C	15 min

b. Refer to the table below for thermocycling conditions (Table 9).
c. Purify the amplified PCR products using the QIAquick PCR Purification Kit (Qiagen), following the instructions recommended by the manufacturer and elute in 35 μL ddH_2O.

21. Second nested PCR.
    a. Perform second round amplification using the Q5 Hot Start High Fidelity DNA Polymerase and the primer pairs P5B5 and P7. Refer to the table below for PCR mixture recipe (Table 10).
    b. Refer to the table below for thermocycling conditions (Table 11).
    c. Purify the amplified PCR products using the QIAquick PCR Purification Kit (Qiagen) according to the instructions provided by the manufacturer and elute in 35 μL ddH_2O.
    d. Run 2 μL purified PCR product on a 2% agarose gel (Figure 2C).
22. LM-PCR library sequencing:
    a. Prepare the LM-PCR library according to Denature and Dilute Library Guide for MiSeq system by Illumina-MiSeq (Document # 15039740v10) and load the samples in the designated sample lading well of "Paired-End Reagent Plate".
    b. Before initiating the sequencing process, add the primers into the specified wells of the "Paired-End Reagent Plate" based on the information provided in Table 12. Mix the primers slowly 3 to 5 times using long tips.
    c. Perform the sequencing run in paired-end mode with a minimum read length of 50 cycles for each read.

EXPECTED OUTCOMES

As shown in Figure 3A, monotreatment with LRA (especially PKC activator, PEP005) did not suppress HIV-1 replication during the first 9 weeks of cultivation, while ART (especially the NRTI, EFdA/islatravir) alone successfully decreased viral production in the supernatant to undetectable levels after 4–6 weeks. The combination treatment of ART and LRA also reduced the HIV-1 level in the supernatant to undetectable levels. Importantly, cessation of treatment at week 9 resulted in a significant rebound of viral production in samples previously treated with ART alone. On the other hand, no such rebound was noted in samples treated with the ART plus LRA combination.

To evaluate the effect of LRA on elimination of latent cells, we analyzed cultures of HIV-1-latently infected cells during the initial phase of the drug treatment. HIV flow cytometry showed that EFdA did not induce marked changes in the proportion of latently infected cells during the first 4 weeks of treatment, compared with the rapid decrease in these cells when treated with the EFdA+LRA

Table 8. PCR mixture for first External PCR

Reagents	Volume/well
P5B5 primer (10 μM)	2 μL
P7 primer (10 μM)	2 μL
dNTP mix (10 mM)	1 μL
Q5 Reaction Buffer 5×	10 μL
Q5 Hot Start High Fidelity DNA Polymerase	0.5 μL
Nuclease free water	14.5 μL
Sample [From step 17 (i)]	20 μL
Final volume	50 μL

Table 9. Thermocycling conditions for the first external PCR

Temperature	Time	Cycles
96°C	30 s	1
94°C	5 s	7
72°C	1 min	
94°C	5 s	13
68°C	1 min	
68°C	9 min	1
4°C	∞	Hold

combination (Figure 3B). It is possible that LRA shortened the time required for activation of latently infected cells, and thus enhanced the process of apoptosis.

LIMITATIONS

The study has certain limitations. First, in our infection and treatment model, elimination of HIV-1 latently infected cells occurs only through apoptosis or cytopathic effect elicited by reactivation, since the model does not include antiviral cytotoxic T lymphocytes. This is clearly different from the HIV infection in human. Second, the results of the WIPE assay are affected by the initial number of latent cells. It is important to check the baseline level of provirus DNA and the proportion of latently infected cells by HIV flow cytometry. Importantly, the outcome of treatment is also influenced by the type of ART used. In this regard, the use of less active NRTIs (*e.g.*, FTC, 3TC) tends to hasten any rebound after discontinuation of the treatment.

TROUBLESHOOTING

Problem 1

Decrease in the supernatant HIV viral titer during long-term assay even in the absence of treatment (Steps 7–8).

Potential solution

Measure p24 amount in the supernatants of drug-free cultured cells. If the amount of p24 is lower than that of the baseline, quantify the number (percentage) of intracellular $p24^+$ cells using flow cytometry. If the percentage is low (<1%), terminate the assay and redo the assay using a newly established HIV-1-latently infected cells.

Problem 2

No or multiple band(s) in the near-full-length single genome PCR (Step 14).

Potential solution

Increase or reduce the amount of DNA used in the first-round PCR.

Problem 3

ddPCR not enough separation between positive and negative dots (Step 11).

Table 10. PCR mixture for second round amplification

Reagents	Volume/well
B3 primer (10 μM)	2 μL
B4 primer (10 μM)	2 μL
dNTP mix (10 mM)	1 μL
Q5 Reaction Buffer 5×	10 μL
Q5 Hot Start High Fidelity DNA Polymerase	0.5 μL
Nuclease free water	32.5 μL
Sample [From step 18(l)]	2 μL
Final volume	50 μL

Table 11. Thermocycling conditions for the second nested PCR

Temperature	Time	Cycles
96°C	30 s	1
94°C	5 s	7
72°C	1 min	
94°C	5 s	13
68°C	1 min	
68°C	9 min	1
4°C	∞	Hold

Potential solution

Please optimize ddPCR condition by changing primer concentration, amount of input DNA, or labeling fluorophores. In our experience, current protocol was the best with Bio-Rad machine.

Problem 4

DNA sonication size differs from expected (Step 15).

Potential solution

If you see shorter or longer fragment size then expected after sonication, please optimize sonication condition. Alternatively, there is an option to use enzymatic fragmentation.

Problem 5

Not enough integration site (IS) event in LM-PCR (Step 17–21).

Potential solution

1. Different temperature control among different PCR machines.
   a. Each PCR machine has different temperature control. That can make the different outcome of LM-PCR.
   b. In our experience, we used LifeEco PCR system (Model TC-96) (HangZhou Bioer Technology) and generally get good IS detection sensitivity. If different PCR machines used, you may need to optimize PCR machine setting.
2. Mismatch between LM-PCR primer and virus used in the assay.
   a. Please use identical primer sequence with the virus used in the WIPE assay.
   b. There are some different sequences in the LTR among various laboratory HIV-1 strain, so mismatch between LM-PCR primer and virus used in the assay can induce inefficient amplification. That would underestimate integration site event in the LM-PCR.

Problem 6

Over clustering in LM-PCR sequencing (Step 22).

Potential solution

1. If Illumina MiSeq platform is used for sequencing LM-PCR reads, it is advisable to decrease the loading library concentration by 20%–40% below the optimal range specified by the chemistry version of the sequencing kit to reduce the chance of over-clustering.

Table 12. Sequencing primer description

Sl.	Primer	Description	Designated well number	Strength	Amount
1.	Sequencing primer for LM-PCR	'Read1' targeting HIV-1	12	10 μM	34 μL
2.	Sequencing primer for LM-PCR	'Index1' targeting adapter barcode	13	10 μM	34 μL
3.	Sequencing primer for LM-PCR	'Read2' targeting human genome	14	10 μM	34 μL

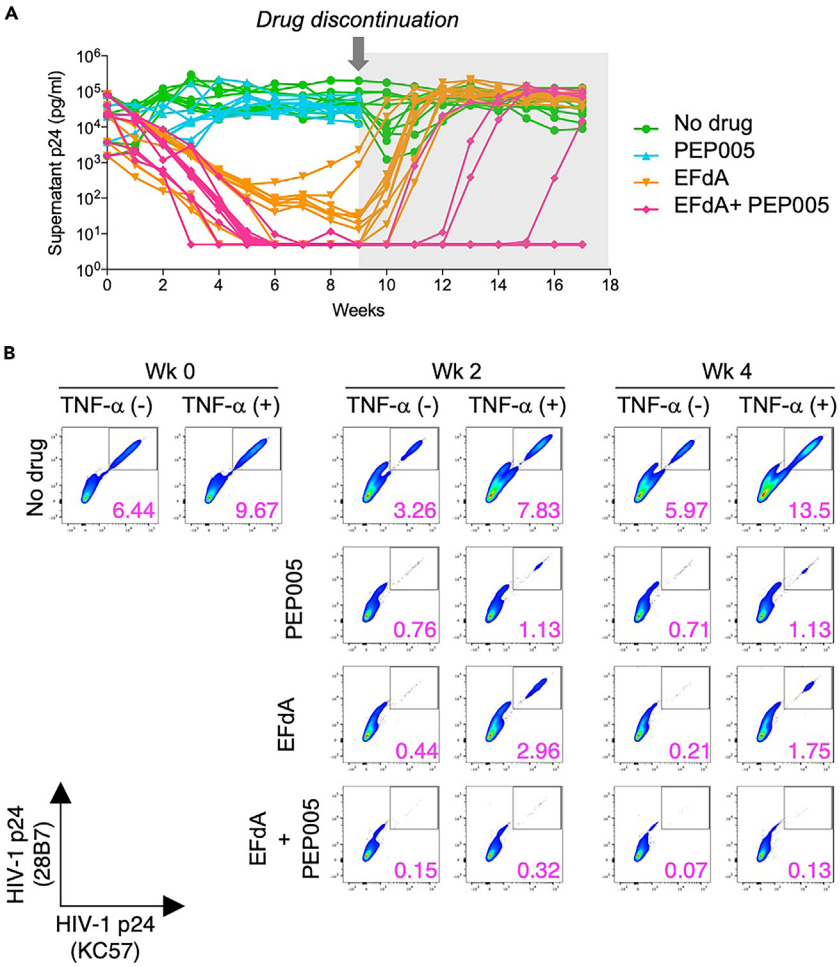

Figure 3. Effects of different drugs on viral persistence by the WIPE assay

(A) Changes in supernatant p24 levels cultured in the absence of drugs, in the presence of 5 nM PEP005 or 50nM EFdA, or with the combination of 50 nM EFdA plus 5 nM PEP005 (n = 11, 9, 11, and 11, respectively). Drug treatment was terminated on week 9, but analysis continued for additional 8 weeks.

(B) Changes in cell population during the early phase of treatment. The percentage of HIV-1-double positive (DP) cells was determined. Cell samples from the WIPE assays (weeks 0, 2 and 4) and those after 24-h TNF-α stimulation was examined after p24-double staining by flow cytometry. Panels form this figure are reused with permission from all authors from our previously published paper.[1]

2. To enhance the diversity of sequence reads, it is recommended to include a 30% PhiX spike-in during sample loading.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to the lead contact, Kenji Maeda (kmaeda@kufm.kagoshima-u.ac.jp).

Materials availability

This study did not generative new unique reagents.

Data and code availability

The published article includes all datasets generated or analyzed during this study.

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AUTHOR CONTRIBUTIONS

Y.S. and Ke.M. designed the study. Ko.M. performed the experiments. B.J.Y.T., S.A.R., and Y.S. performed bioinformatic analysis. K.T., Y.S., and Ke.M. supervised the work. Ko.M. and Ke.M. wrote the manuscript with input from all other authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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