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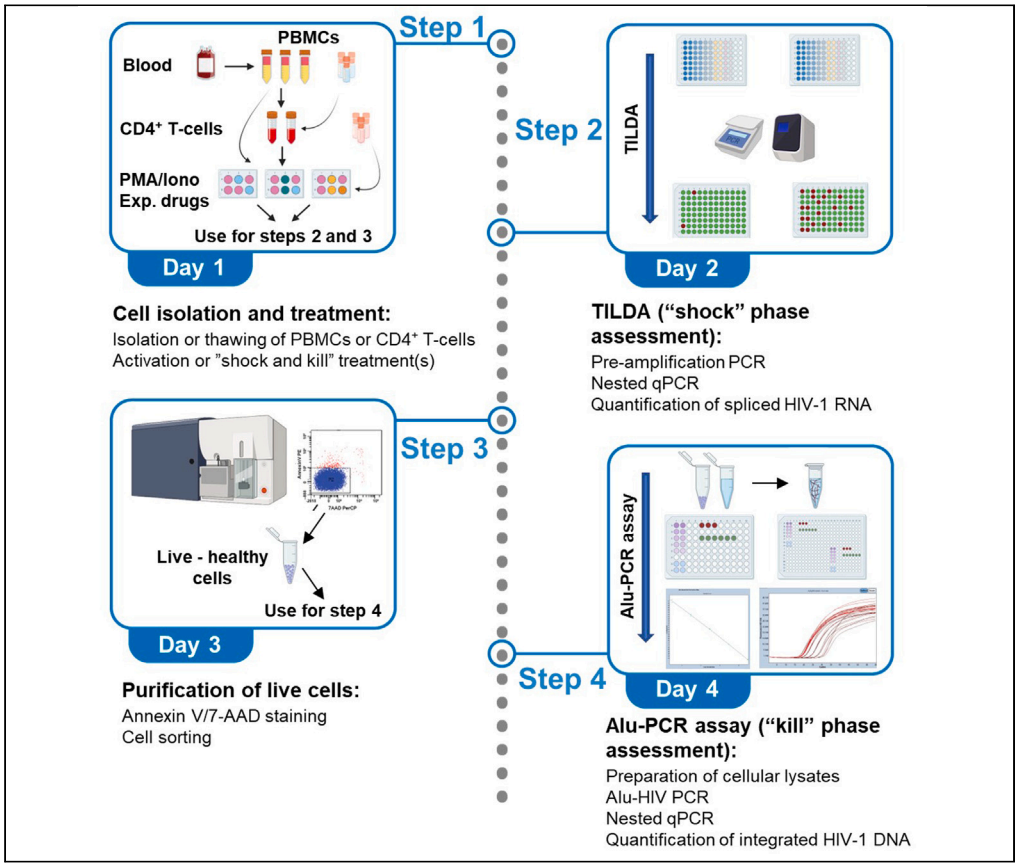
A scalable workflow to test "shock and kill" therapeutic approaches against the HIV-1 latent reservoir in blood cells *ex vivo*



Integrated HIV-1 DNA persists in cells of people living with HIV during antiretroviral treatment, but its quantification is hindered by its rarity. Here, we present an optimized protocol to evaluate "shock and kill" therapeutic strategies, including both the latency reactivation ("shock") and elimination of infected cells ("kill") stages. We describe steps for the sequential use of nested PCR-based assays and viability sorting to allow for scalable and rapid screening of candidate therapeutics in patient-derived blood cells.

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

Francesco Andrea Procopio, Andrea Savarino, Iart Luca Shytaj

francesco.procopio@chuv.ch (F.A.P.)
luca.shytaj@bristol.ac.uk (I.L.S.)

Highlights

Quantitative assessment of novel therapeutics to target latently HIV-1-infected cells

Workflow optimized for patient-derived cells

Same-sample estimate of both latency reactivation and infected cell killing

Protocol suitable for low-cell inputs

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A scalable workflow to test ''shock and kill'' therapeutic approaches against the HIV-1 latent reservoir in blood cells *ex vivo*

Francesco Andrea Procopio,[1,4,*] Andrea Savarino,[2] and Iart Luca Shytaj[3,5,*]

[1]Services of Immunology and Allergy, Lausanne University Hospital, University of Lausanne, 1011 Lausanne, Switzerland

[2]Department of Infectious Diseases, Italian Institute of Health, 00161 Rome, Italy

[3]Faculty of Life Sciences, School of Cellular and Molecular Medicine, University of Bristol, BS8 1TD Bristol, UK

[4]Technical contact

[5]Lead contact

*Correspondence: francesco.procopio@chuv.ch (F.A.P.), luca.shytaj@bristol.ac.uk (I.L.S.)
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SUMMARY

Integrated HIV-1 DNA persists in cells of people living with HIV during antiretroviral treatment, but its quantification is hindered by its rarity. Here, we present an optimized protocol to evaluate ''shock and kill'' therapeutic strategies, including both the latency reactivation (''shock'') and elimination of infected cells (''kill'') stages. We describe steps for the sequential use of nested PCR-based assays and viability sorting to allow for scalable and rapid screening of candidate therapeutics in patient-derived blood cells.

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

BEFORE YOU BEGIN

HIV eradication is an unmet medical need, due to persistence of the virus in a non-expressing, integrated form in the so called retroviral ''reservoirs'', mainly memory $CD4^+$ T-cells.[2] One popular wave of research aimed at eradicating HIV is the ''shock and kill'' approach, i.e., reversing HIV latency through drugs or other stimuli (''shock'' phase) followed by cytotoxic stimuli selectively exploiting fingerprints of retroviral replication, both antigenic and metabolic (''kill'' phase).[3]

The present workflow describes the specific steps for using PLWH-derived $CD4^+$ T cells to evaluate the efficacy of both phases of ''shock'' and ''kill'' treatments. The outcome of the ''shock'' phase is analyzed by quantifying HIV-1 RNA transcriptional activity with the TatRev-Induced-Limiting-Dilution assay (TILDA), while the ''kill'' phase is evaluated by quantifying the integrated HIV-1 DNA content with the Alu-PCR assay. The same workflow can be used for peripheral blood mononuclear cells (PBMCs) or $CD4^+$ T cells isolated from other sources, such as lymph nodes and gut associated lymphoid tissue (GALT). In theory, the protocol herein described can be used for any cell population supporting HIV-1 integration and replication. However, it is important to note that the use of PBMCs might lead to more false negative results due to the low frequency of latently infected cells in the whole PBMC population and to the possible inhibition of HIV reactivation by $CD8^+$ T-cells.[4]

The TILDA assay was previously described[5] and uses serial dilutions to detect spliced viral mRNA transcripts (i.e., Tat and Rev), yielding fast and precise quantification of the HIV-1 nucleic acids using low target cell inputs (< than 1×10^6).

The Alu-PCR protocol is instead an adaptation of Vandergeeten et al.[6] This optimized protocol replaces the DNA extraction with proteinase K (pK) digestion to remove protein content and prevent nucleic acid degradation, thereby increasing scalability and reducing cost. To this purpose, cells are first digested with pK and the cell lysate is then directly used to perform a first round of amplification (pre-amplification) of the Alu-HIV-1 LTR amplicon and of the host's CD3 gene. The pre-amplification product is then used as template for a nested real-time PCR using TaqMan probes. Moreover, cell sorting for viability prior to the Alu-PCR protocol is here applied to decrease possible noise signals in experiments testing therapeutic interventions with selective cytotoxic potential.

Although scalable protocols had been published to measure the "shock" phase of the approach in patient- derived cells,[7–9] the current workflow is the first detailed description of a scalable method recently applied to test both the "shock" and the "kill" phases of the approach under conditions as close as possible to an *in vivo* setting.[1]

Institutional permissions

All experiments on primary blood cells can be performed solely after approval by the relevant ethics committees and in accordance with national guidelines and regulations. Moreover, all experiments using infectious material must be performed in accordance with approved risk-assessment protocols and at the biosafety level prescribed by local regulations.

Preparation of R10 and R20 media solutions

Timing: 5 min

1. Warm up, to 37°C for 30 min, the RPMI medium, the penicillin/streptomycin solution and the fetal bovine serum (FBS).
   a. To prepare RPMI with 10% serum (R10), add 50 mL of FBS and 5 mL of penicillin/streptomycin solution to 445 mL RPMI.
   b. To prepare RPMI with 20% serum (R20) add 100 mL of FBS and 5 mL of penicillin/streptomycin solution to a 395 mL RPMI.

Note: FBS must be de-complemented before first use by warming at 56°C for 30 min.

Note: R10 and R20 can be stored at 4°C for up to a month.

Preparation of PMA and ionomycin solutions as a positive control for the "shock" phase

Timing: 20 min

2. PMA and ionomycin need to be both dissolved in DMSO to prepare working solutions.
   a. Resuspend 1 mg ionomycin in 1 mL of DMSO.
   b. In parallel, resuspend 100 μg PMA in another 1 mL of DMSO.
   c. Mix with a 1 mL micropipette until both compounds are fully dissolved.
   d. Prepare 20 μL aliquots of both solutions, and store at −20°C for up to a year.

Preparation of proteinase K (pK) solution

Timing: 30 min

3. Resuspend 100 mg of pK in 10 mL of molecular biology grade H_2O and prepare 400 μL aliquots.
4. To a 400 μL of diluted pK add: 1 mL of 0.1 M TrisHCl (pH 8.0), 1 mL of 0.5 M KCl and 7.6 mL of molecular biology grade H_2O.
5. Prepare 1 mL aliquots.

Pause point: the solutions prepared at steps 3 and 5 can be stored at −20°C for up to a year.

Note: the pK solution should be prepared and stored in a separate space, free from PCR amplicons (e.g., pre-PCR room).

Preparation of Alu-PCR standard for measuring the "kill" phase

Timing: 13–15 days

The standard is prepared using ACH-2 cells, a lymphoid, non-adherent cell line latently infected with HIV-1 and harboring a single copy of integrated HIV-1 DNA.[10]

6. Expand the ACH-2 cell line culture and prepare the first ACH-2 standard.
   a. Transfer a vial of cryopreserved ACH-2 cells (generally 10 × 10^6 cells per vial) to a 37°C water bath and wait until the vial is completely thawed.
   b. Transfer the content of the vial to a 50 mL conic tube and add, drop by drop, 40 mL of R20 media solution.
   c. Centrifuge at 600 × *g* for 10 min at 15°C–25°C and discard the R20 media solution supernatant.
   d. Add 10 mL of R10 media solution.
   e. Centrifuge at 600 × *g* for 10 min at 15°C–25°C and discard the supernatant.
   f. Add 10 mL of R10 media solution and transfer the cells in a 25 cm^2 flask.
   g. Keep cells in an incubator at 37°C, 5% CO_2 for 3–5 days.
   h. Transfer the flask content into a 50 mL tube and add 40 mL of R10 media solution.
   i. Pellet the cells by centrifuging at 600 × *g* for 10 min at 15°C–25°C.
   j. Resuspend the cells in 10 mL of R10 media solution and transfer them to a new 25 cm^2 flask.
   k. Keep cells in an incubator at 37°C, 5% CO_2 for 7 days.
   l. Transfer the flask content into a 50 mL tube and add 40 mL of R10 media solution.
   m. Pellet the cells by centrifuging at 600 × *g* for 10 min at 15°C–25°C.
   n. Resuspend the cells in 10 mL of R10 media solution.
   o. Count the cells with your method of choice.
   p. Resuspend the cells at a concentration of 10^6 cells/mL in R10 media solution and prepare multiple 1 mL aliquots of the cell suspension in 1.5 mL tubes.
   q. Centrifuge the cells at 600 × *g* for 10 min at 15°C–25°C and remove the supernatant to obtain pellets.
   r. Add 500 µL of pK solution to a 1 × 10^6 pellet aliquot and incubate at 55°C for 16–24 h in a thermos-shaker or waterbath.
   s. Vortex the vial a few times every hour for the first 3 h of incubation or, if using a thermos-shaker, set a gentle agitation (e.g., 50 × *g*).
   t. The day after, set a thermos block at 95°C and incubate the vials for 5 min.
   u. Split the 500 µL content of each vial in multiple 30 µL aliquots in 1.5 mL tubes.

⚠ CRITICAL: the quality of the standard will mostly depend on the precision of the ACH-2 counting process (step o). We typically use a disposable counting chamber slide and base the count on a minimum of 2 slides.

Pause point: the pellets obtained at step i and the aliquots at step u can be stored at −20°C for up to one and two years, respectively.

Note: while the HIV-1 DNA standard could be generated also using a plasmid, the use of an infected cell line with predictable integrated HIV-1 DNA content may mimic more closely the conditions expected in the experimental samples.

Note: although the viability of the culture could also be assessed during step b (e.g., trypan blue solution), this is usually not essential as it is expected that only a small number of cells will be dying/dead and that they will be eliminated during the centrifugation in the following steps.

Note: at step k, cells should be in the exponential growth phase, doubling on average every 24 h and appearing round and ≈ 3–5 times larger (in diameter) than activated primary T cells (5–10 μm).

Note: steps r-u can be repeated for multiple aliquots at the same time. This will reduce experimental variability.

Note: At the end of the procedure (i.e., after step u) 15 μL of solution contains 30,000 cells, equivalent to 30,000 copies of the HIV-1 DNA genome.

Primers and probes preparation

Timing: 10 m

7. Spin lyophilized primers and probes at 20,000 × *g* or maximum speed for a minute.
8. Resuspend primers and probes at 100 μM stock concentration.
9. Dilute to 20 μM (primers) or 10 μM (probes) to obtain working stocks.

Note: primers and probes can be ordered from any supplier, but we suggest Integrated DNA Technologies (IDTDNA; Coralville, Iowa USA). The probes can be ordered with any reporter and quencher (e.g., the classic 5′FAM-3′TAMRA). We recommend using a double quencher probe (e.g., ZEN / Iowa Black FQ *) to reduce the background.

Pause point: after resuspension (step 8), primers and probes can be stored at −20°C for up to a year with no efficiency loss.

FACS cell sorter startup and setup

Timing: 1 h

The cell sorting step is important to assess the "kill" phase of the workflow. The following steps are required to setup a FACSAria II cell sorter, the reader should adapt these indications to their specific instrument/model.

10. Define the baseline performance of the cytometer by loading a tube filled with 1 drop of Cytometer Set-Up and Tracking (CS&T) beads in 1 mL of sheath fluid.
11. Set-up the experiment using the Accudrop beads.
    a. Select the appropriate sorting nozzle (generally between 70-100 microns).
    b. Load a tube filled with 2 drops of Accudrop beads in 1 mL of sheath fluid.
    c. Setup of the droplet stream into the chosen collection tubes or plates.
12. Perform the 8-peak analysis loading a tube containing 1 drop of 8-peak beads in 1 mL of sheath fluid.

Note: the startup and setup of any cell sorter involves a complex process, generally performed by a trained operator. For our experiments, we used a BD FACSAria II cell sorter, although any similar instrument could alternatively be integrated in the same workflow.

Note: in step 10 parameters related to linearity, detector efficiency, electronic noise, and laser delays, among others, are evaluated. PMT (photo multiplicators) voltages are adjusted to maximize population resolution in each detector and to evaluate the consistency of the instrument parameters overtime.

Note: step 11 is important to ensure that the correct droplets (single-cell droplets) are sorted.

Note: step 12 uses 8-peak beads (also known as "rainbow" beads) which are a set of beads containing eight different populations that differ exclusively in their amount of fluorophore. The first peak is generally unlabeled, and the remaining ones contain increasing amounts of fluorophore. The rainbow beads fluoresce in all channels and are used to check fluorescence sensitivity and resolution by measuring the position of the unlabeled peak and the separation between all peaks. These beads are also necessary to check linearity in the fluorescence detection channel.

Annexin V binding buffer solution

Timing: 5 min

13. Prepare a 1× working solution by diluting the 10× Annexin V Binding Buffer (available in the Annexin V/7-AAD kit) in distilled H_2O at a 1:10 ratio.

Note: the 1× Annexin V Binding Buffer solution can be stored at 4°C for up to 6 h.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Whole blood (or buffy coat)	PLWH or optionally healthy donors (irrespective of age and gender)	N.A.
Chemicals, peptides, and recombinant proteins		
Phorbol 12-myristate 13-acetate (PMA) 1 mg	Sigma-Aldrich	P1585-1mg
Ionomycin from *Streptomyces conglobatus* 1 mg	Sigma-Aldrich	I9657
Ficoll® Paque Plus	Sigma-Aldrich	GE17-1440-02
PBS (pH 7.4)	Thermo Fisher	10010023
S1810 - FETAL BOVINE SERUM (FBS) SOUTH AMERICA	Biowest	s1810-500
Penicillin/streptomycin 100 mL solution (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin)	BioConcept	4-01f00-h
TrisHCl pH8 1 M	Thermo Fisher	15568025
KCl 2 M	Thermo Fisher	AM9640G
TE buffer, pH 8.0, RNase-free (10 mM Tris, 1 mM EDTA)	Thermo Fisher	AM9858
RPMI 1640 Medium, GlutaMAX™ Supplement	Thermo Fisher	61870-010
SUPERase-In Rnase inhibitor (20 U/µL)	Thermo Fisher	AM2694
Proteinase K, recombinant (pK) 100 mg	Thermo Fisher	25530015
Critical commercial assays		
EasySep™ Human CD4+ T Cell Isolation Kit	STEMCELL Technologies	17952
FastStart essential DNA probes master	Roche	6402682001
SuperScript™ III Platinum™ One-Step qRT-PCR Kit w/ROX	Thermo Fisher	11745100
Taq DNA Polymerase, native	Thermo Fisher	18038018
CS&T Research Beads	BD Biosciences	655050
Accudrop beads	BD Biosciences	345249

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Rainbow Calibration Particles (8 peaks)	BD Biosciences	559123
PE Annexin V Apoptosis Detection Kit I	BD Biosciences	559763
Experimental models: Cell lines		
ACH-2 cell line	NIH AIDS Reagent Program (acute lymphoblastic leukemia from 4-years old female)	ARP-349
Oligonucleotides		
TILDA tat1.4: 5′-TGG CAG GAA GAA GCG GAG A-3′	Procopio et al.[5]	
TILDA rev: 5′-GGA TCT GTC TCT GTC TCT CTC TCC ACC-3′	Procopio et al.[5]	
TILDA tat2: 5′- ACA GTC AGA CTC ATC AAG TTT CTC TAT CAA AGC A -3′	Procopio et al.[5]	
TILDA probe 5′-/56-FAM/TTC CTT CGG /ZEN/GCC TGT CGG GTC CC/3IABkFQ/-3′	Procopio et al.[5]	
HCD3 out 5′: 5′-ACT GAC ATG GAA CAG GGG AAG-3′	IDTDNA	
HCD3 out 3′: 5′-CCA GCT CTG AAG TAG GGA ACA TAT-3′	IDTDNA	
HCD3 in 5′: 5′-GGC TAT CAT TCT TCT TCA AGG T-3′	IDTDNA	
HCD3 in 3′: 5′-CCT CTC TTC AGC CAT TTA AGT A-3′	IDTDNA	
ULF1: 5′-ATG CCA CGT AAG CGA AAC T CT GGG TCT CTC TDG TTA G AC-3′	IDTDNA	
Alu1: 5′-TCC CAG CTA CTG GGG AGG CTG AGG-3′	IDTDNA	
Alu2: 5′-GCC TCC CAA AGT GCT GGG ATT ACA G-3′	IDTDNA	
LambdaT: 5′-ATG CCA CGT AAG CGA AAC T -3′	IDTDNA	
UR2: 5′- CTG AGG GAT CTC TAG TTA CC-3′	IDTDNA	
CD3 probe: 56FAM 5′-AGCAGAGAAZENCAGT TAAGAGCCTCCAT-3′ 3IABkFQ	IDTDNA	
HIV probe: 56FAM 5′-CACTCAAGGZENCAAG CTTTATTGAGGC-3′ 3IABkFQ	IDTDNA	
Software and algorithms		
Extreme Limiting Dilution Analysis (ELDA)	WEHI Bioinformatics	https://bioinf.wehi.edu.au/software/elda/
BD FACSDiva™ software v7	BD bioscience	N.A.
Other		
Costar® 24-well Clear TC-treated Multiple Well Plates, Individually Wrapped, Sterile	Costar	3524
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	Thermofisher	4346906
Adhesive PCR Plate Seals	Thermofisher	AB-0558
LightCycler® 480 Multiwell Plate 384, white	Roche	04729749001
Thermo Scientific™ ABsolute qPCR Plate Seals	Thermofisher	AB-1170
PCR instrument: Verity 96 wells fast thermal cycler	Thermofisher	4375305
Real-time PCR instrument: LightCycler 480II	Roche	05 015 243 001
EasySep Magnet	Stemcell Technologies	05015234001
RNase AWAY™ Surface Decontaminant	Thermofisher	7003
Eppendorf Mastercycler Vapo Protect Thermocycler 6325	Eppendorf	6325cl218283
FACSAria IIu	BD bioscience	N.A.
Countess™ Cell Counting Chamber Slides	Thermofisher	C10228

MATERIALS AND EQUIPMENT

PMA/Ionomycin activation solution (for 50 × 10^6 cells)		
Component	Final concentration	Volume
R10 complemented medium	1×	50 mL
PMA in DMSO (100 µg/mL)	100 ng/mL	50 µL
Ionomycin in DMSO (1 mg/mL)	1 µg/mL	50 µL

Proteinase K (pK) solution		
Component	Final concentration	Volume
pK in molecular biology grade H_2O	0.4 mg/mL	400 µL of 10 mg/mL solution
0.1 M TrisHCl (pH 8.0)	0.01 M	1 mL
0.5 M KCl	0.05 M	1 mL
molecular biology grade H_2O	N.A.	7.6 mL

Storage condition.
R10 complemented medium can be stored at 4°C for up to a month.
PMA in DMSO, Ionomycin in DMSO, pK in H_2O, and the pK solution can be stored at −20°C for up to a year. TrisHCl and KCl can be stored at 15°C–25°C for up to a year.
The PMA/Ionomycin activation solution is not stored (prepare an appropriate amount for single use).

STEP-BY-STEP METHOD DETAILS

Day 1

$CD4^+$ T-cell separation, cell activation and drug testing

Timing: 6 h

During the first day, $CD4^+$ T-cells (or PBMCs) will be separated from whole blood of PLWH or thawed from frozen aliquots. The $CD4^+$ T cell separation is based on antibody labeling of all PBMC sub-populations except our cells of interest. The antibody-labeled cells can thus be removed using a magnet separator leaving a highly pure $CD4^+$ T cell suspension for downstream use.

Cells will then be activated and/or treated with experimental compounds to assess HIV-1 reactivation for TILDA ("shock" phase) or sorted to isolate live cells for quantification of integrated HIV-1 DNA by Alu-PCR ("kill" phase).

1. Separate PBMCs from fresh blood or leukapheresis using Ficoll Hypaque density gradient centrifugation or using PBMCs cryopreserved in liquid nitrogen.
   a. When starting from whole blood.
      i. Dilute blood 1:1 with PBS.
      ii. Transfer 12 mL of Ficoll Hypaque in a 50 mL conic tube.
      iii. Add slowly 30 mL of diluted blood on the top of the Ficoll solution with the help of a pipette.
      iv. Centrifuge for 20 min at 4°C at 700 × *g* (setting no break in the centrifugation).
      v. Aspirate with a pipette the white/reddish cell ring containing the PBMCs.
   b. When using cryopreserved PBMCs.
      i. Transfer a vial of cryopreserved PBMCs (generally 10–50 million per vial) in a 37°C water bath and wait until the vial is completely thawed.
      ii. Transfer the content of the vial to a 50 mL conic tube.
      iii. Add, drop by drop, 40 mL of R20 media solution.
      iv. Centrifuge at 600 × *g* for 10 min.
      v. Discard the R20 media solution and add 40 mL of R10 media solution.
      vi. Centrifuge at 600 × *g* for 10 min.
      vii. Discard the R10 media solution and add another 10 mL of R10 media solution.
      viii. Count cells.
2. Isolate $CD4^+$ T-cells by negative magnetic selection.
   a. Warm the negative magnetic selection kit to 15°C–25°C at least 30 min before use.
   b. Resuspend 50 million PBMCs in 1 mL of R10 media solution and transfer to a 5 mL conic polystyrene tube.
   c. Add 50 µL of the negative selection antibody solution (provided with the negative selection kit) and incubate for 5 min.

d. Right before use, vortex the magnetic beads present in the negative selection kit and add 50 μL of magnetic bead preparation to the cell suspension.
e. After a 5 min incubation, add an additional 2 mL of R10 to the 5 mL conic polystyrene tube and mix the solution with the help of a pipette.
f. Add 50 μL of magnetic bead suspension and again mix a few times with the help of a pipette.
g. Transfer the tube into the magnetic separator and incubate for 5 min.
h. Invert the magnet and the tube pouring the negatively selected cells into a new tube.
i. Count cells.

Note: we suggest always assessing the $CD4^+$ T-cell purity by FACS analysis and, in case it should be lower than 95%, repeating the $CD4^+$ T-cell negative selection process. When thawed cells are used, it is also particularly important to check for the presence of dead cells and exclude them from the counting process (e.g., using trypan blue).

⚠ CRITICAL: at least 2×10^6 $CD4^+$ T-cells per experimental condition (i.e., resting, activated with or without the experimental drugs to be tested) are required for the whole workflow, with 1×10^6 set aside for TILDA and 1×10^6 for sorting viable cells and downstream Alu-PCR. These amounts can be typically obtained from 20×10^6 PBMCs or 10 mL of total blood. When possible, it is recommended to start from larger volumes/amounts to guarantee the possibility of including additional replicates of the same experimental condition.

3. Activate isolated $CD4^+$ T-cells (or PBMCs) to provide a positive control for the TILDA assay and/or treat them with experimental compounds to test their "shock and kill" effects.
   a. Transfer 1 mL of $CD4^+$ T-cells at a concentration of 2×10^6 cells/mL in R10 media solution to a 24-well plate.
   b. Keep cells in an incubator at 37°C, 5% CO_2 for 3–5 h.
   c. Add 1 μL of PMA and 1 μL of ionomycin solution per each mL of cell suspension (final concentrations: 100 ng/mL PMA and 1 μg/mL ionomycin).
   d. Incubate cells with PMA and ionomycin for 12–18 h to have a positive control for the reactivation of the HIV-1 reservoir.
   e. Split the remaining cells in the required conditions for treatment with the experimental drug(s) of choice, leaving at least 2×10^6 cells under resting condition as controls.

Note: when $CD4^+$ T-cells are separated from fresh blood, no incubation time is necessary before step 3c (stimulation). Repeated freeze-thaw cycles should be avoided for the PMA or ionomycin working solutions.

Note: assuming for simplicity the testing of a single experimental drug, the minimum number of conditions included in step 3e will be: resting condition (negative control), PMA/ionomycin condition (positive reactivation control), and the experimental drug treatment condition. Additionally, a condition of cells treated with PMA/ionomycin plus the experimental drug can be included in the same setup to evaluate the ability of the experimental drug to boost/inhibit the reactivation potential of other stimuli.

Note: the protocol and the cell quantities that we recommend are set on an ideal "shock and kill" protocol, in which one drug provides both the "shock" and the "kill" stimulus. However, it is also possible, and likely, that two or more drugs will be required, each providing either the stimulus to reactivate HIV-1 from latency or to induce selective killing of the infected $CD4^+$ T-cells. If two or more drugs are used, the number of treatment conditions will depend on whether the "kill" drug is expected to exert an effect *per se* ("silent killing" of the latently infected cells without HIV-1 reactivation) or whether its effects are dependent on the previous viral reactivation. In the latter case, testing only the drug combination may be sufficient. Overall, although the total amount of cells to allocate for step 3e will depend on the number of

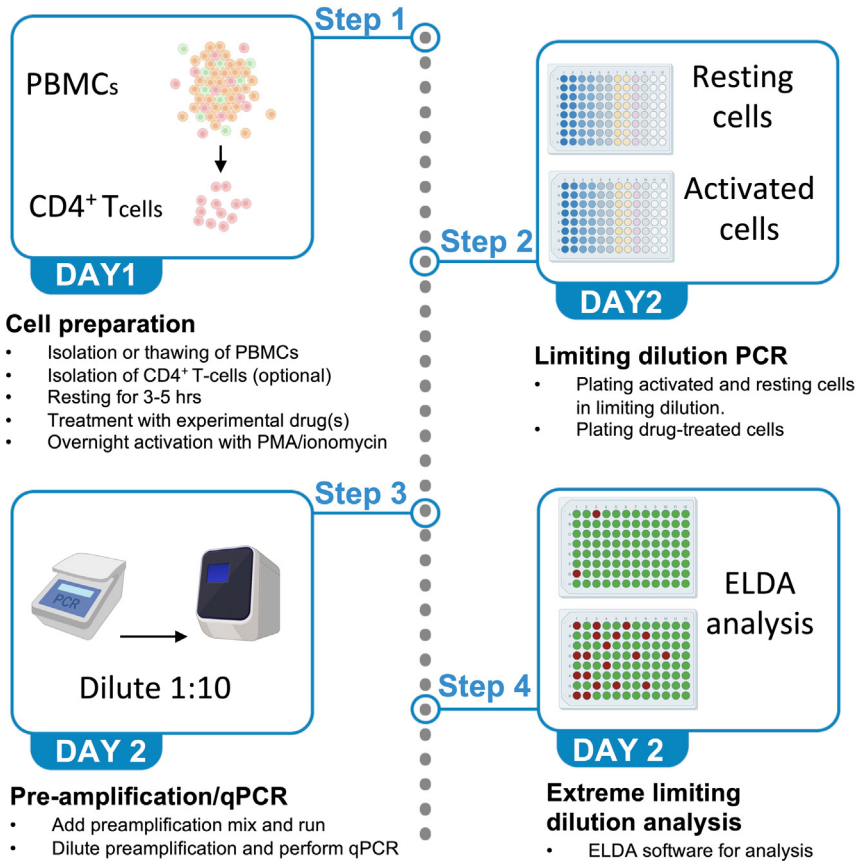

Figure 1. Schematic workflow of TILDA

TILDA is used to evaluate the "shock" phase (HIV-1 latency reactivation) by performing a nested PCR of a serially diluted cell input to detect spliced HIV-1 mRNA transcripts (i.e., Tat and Rev). After isolation from whole blood of PLWH, target cells (typically $CD4^+$ T-cells or PBMCs) are left untreated or activated with PMA/ionomycin or treated with experimental compound(s) (step 1). Cells are then plated at serial dilutions (as detailed in the main text) and used for the pre-amplification PCR (step 2). The pre-amplificates are then loaded in a qPCR assay (step 3) and the expression of spliced HIV-1 mRNA is calculated with a dedicated software (step 4).

experimental drugs/concentrations envisaged for the experiment; even in the case of experiments testing multiple drugs, we recommend plating at least 2×10^6 cells per condition.

Day 2

TILDA preamplification, nested-qPCR and analysis ("shock" phase)

Timing: 7 h

During the second day the cells will be probed for the presence of spliced HIV-1 mRNA using TILDA (the procedure of which is summarized in Figure 1). This is accomplished by performing a pre- amplification (first PCR) followed by a nested qPCR (second PCR) on cells serially diluted in a 96-well plate. A limiting dilution calculator software will finally be used to estimate the number of cells expressing spliced HIV-1 mRNA.

4. Prepare the pre-amplification (first PCR) mix.
   a. Follow the calculations shown in Table 1 below to prepare the pre-amplification (first PCR) mix.
   b. Distribute the pre-amplification mix adding 10 µL per well in the pre-amplification plate.
5. Prepare the nested qPCR mix.

Table 1. Calculations for master mix preparation of the pre-amplification PCR (first PCR) of TILDA

Reagent	Final concentration (in 11 μL)	Amount
2× reaction buffer	1×	5.5 μL
Superscript III/Platinum Taq	N.A.	0.2 μL
RNase inhibitor (20 U/μL)	0.18 U/μL	0.1 μL
primer tat1.4 (20 μM)	227 nM	0.125 μL
primer rev (20 μM)	227 nM	0.125 μL
TE buffer	N.A.	2.2 μL
ddH_2O	N.A.	1.75 μL
total		**10 μL**

Amounts are calculated for one well.

a. Follow the calculations shown in Table 2 below to prepare the nested qPCR (second PCR) mix.
b. Distribute the nested qPCR mix adding 9 μL per well in the nested qPCR plate(s).

Note: generally, a full 96-well plate is loaded with the nested qPCR mix. In this case it is helpful to prepare a mix for 110 wells and distribute 9 μL per well with a multichannel pipette. The assay is, however, also compatible with 396-well plates which can be useful when multiple samples/conditions are tested.

Note: after distributing the pre-amplification (first PCR) mix and the nested qPCR mix in their allocated plates keep the plates at 4°C until use.

6. Load serial cell dilutions in the pre-amplification plate (first PCR) according to the scheme shown in Figure 2 and following the steps below.
   a. Collect at least 1 × 10^6 $CD4^+$ T-cells (or PBMCs) previously stimulated with PMA/ionomycin (or unstimulated controls, or cells treated with the experimental compounds) in a 1.5 mL tube.
   b. Spin cells for 10 min at 600 × *g*.
   c. Count cells and dilute them in R10 media solution at a concentration of 18 × 10^6 cells/mL (equal to 18,000 cells/μL or 1 × 10^6 $CD4^+$ T cells/55.5 μL). This is referred to as cell component solution A.
   d. Transfer 1 μL of cell component solution A to 24 wells (flat bottom) of the pre-amplification plate (18,000 cells/well).
   e. Transfer 24 μL of cell component solution A to a new sterile 1.5 mL tube and add 24 μL of R10 media solution. This is referred to as cell component solution B.
   f. Transfer 1 μL of cell component solution B to the next 24 wells of the pre-amplification plate (9,000 cells/well).
   g. Add 48 μL of R10 media solution to component solution B. This is referred to as cell component solution C.
   h. Transfer 1 μL of component C to each of the next 24 wells of the pre-amplification plate (3,000 cells/well).

Table 2. Calculations for master mix preparation of the nested qPCR (second PCR) of TILDA

Reagent	Final concentration (in 10 μL)	Amount
Faststart essential DNA probes master 2×	1×	5 μL
primer tat2 (20 μM)	400 nM	0.2 μL
primer rev (20 μM)	400 nM	0.2 μL
FamZen probe (10 μM)	200 nM	0.2 μL
ddH_2O	N.A.	3.4 μL
Total		**9 μL**

Amounts are calculated for one well.

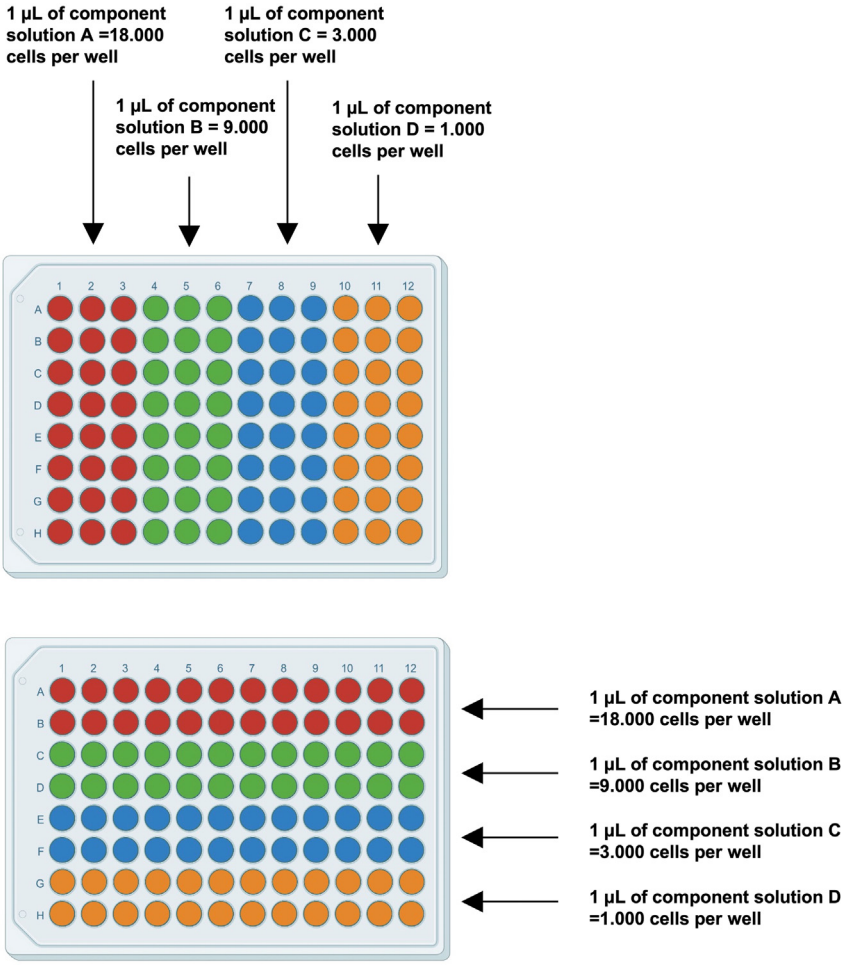

Figure 2. Suggested schemes for plating target cells in limiting dilution in the TILDA pre-amplification plate

The pre-amplification PCR of TILDA uses serial dilutions of target cells to detect spliced HIV-1 mRNA transcripts. The figure depicts typical schemes for plating serial cell dilutions in a 96-well plate. For the specific calculations of the different dilutions (i.e., solutions A-D) refer to step 6 of the "step-by-step method details" section of the manuscript.

i. Add 96 µL of R10 media solution to component solution C. This is referred to as cell component solution D.
j. Transfer 1 µL of component D to each of the next 24 wells of the pre-amplification plate (1,000 cells/well).
k. Seal the pre-amplification plate with a sealing foil, spin the plate at 700 × *g* (or max speed) for 30 s, and transfer the plate to the PCR instrument.

⚠ CRITICAL: to prevent non-specific amplification, keep the plate at 4°C (e.g., cooling holder) until use, also while dispensing cell dilutions.

Note: when collecting cells for TILDA in step 6a, leave at least 1×10^6 cells in culture for downstream analysis (Day 3–4 of the protocol).

Note: the pre amplification reaction requires a full plate for each experimental condition (Figure 3), with a potentially large number of plates required in experimental setups testing multiple drugs and/or drug concentrations. Although plates can be stored for up to 6 h at 4°C until

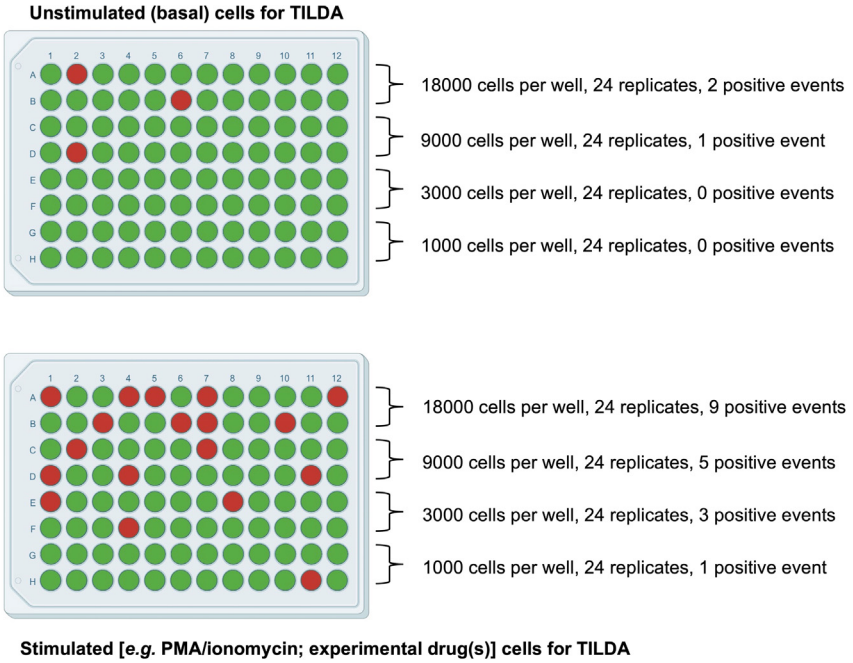

Figure 3. Example of expected qualitative TILDA results

The nested qPCR of TILDA yields an initial qualitative result (i.e., number of wells positive for spliced HIV-1 mRNA transcripts) that can be calculated through the Second Derivative Maximum method or similar methods (step 9 of the main text). Typically, positive wells will be more common in stimulated cells (i.e., cells that were treated with PMA/ionomycin or experimental drugs) and in lower dilution wells.

use, the number of thermocyclers available might be a limiting factor and potentially increase the turnover time of the experiment.

Note: always vortex component solutions A-D (steps 6c-j) for a few seconds before transferring the cells to the pre-amplification plate, to guarantee an even distribution of cells.

Note: the standard TILDA dilution scheme (18,000; 9,000; 3,000; 1,000) can be adapted according to the expected frequency of positive cells. For example, when using cells isolated from the blood of viremic donors (normally harboring a higher number of spliced HIV-1 mRNA positive cells) the scheme can be modified as follows: 9,000; 3,000; 1,000; 333.

Note: if working with low amounts of $CD4^+$ T-cells, the number of replicates per dilution factor (24 × 4) can be scaled. However, this may impact on the accuracy and precision of the measurements (i.e., wider confidence intervals).

7. Start the pre-amplification (first PCR) reaction using the program shown in Table 3 below:
8. Load the nested qPCR plate.

Table 3. Parameters of the pre-amplification PCR (first PCR) for TILDA

Steps	Temperature	Time	Cycles
Reverse transcription	50°C	15 min	1
Denaturation	95°C	2 min	1
Denaturation	95°C	15 s	24 cycles
Annealing and Extension	60°C	4 min	
Hold	4°C	∞	

Table 4. Parameters of the nested qPCR (second PCR) for TILDA

Steps	Temperature	Time	Cycles
Pre-incubation	95°C	10 min	1
Denaturation	95°C	10 s	45 cycles
Annealing and Extension	60°C	30 s	
Final Extension	72°C	1 s	
Cooling	40°C	30 s	
Hold	25°C	∞	

a. At the end of the pre-amplification (first PCR run), carefully remove the sealing foil and add 40 μL of TE buffer 1× to each well of the pre-amplification plate.
b. Mix by gentle pipetting and transfer 1 μL of the diluted PCR products to the equivalent well in the nested qPCR plate.
c. Seal the plate with a qPCR grade sealing foil.
d. Spin the plate at 700 × *g* (or max speed) for 30 s and transfer the plate to the real-time PCR instrument.
e. Start the real time PCR cycling using the program shown in Table 4 below:

9. At the end of the qPCR amplification, perform the initial qualitative analysis using the Second Derivative Maximum method.
   a. Count positive wells at each dilution and record the data (Figure 3).

Note: the Second Derivative Maximum method compares fluorescent curves directly, finding a characteristic (and constant) point on the graph, which reflects the shape of the curve. Alternatively, the Fit Points method, First Derivative method and Cycle Threshold method can be used as well.[11] All these methods are typically incorporated in every modern qPCR instrument software and require little or no input from the operator. Ultimately, the aim of this analysis step is to identify positive and negative wells, rather than comparing the relative amount of spliced HIV-1 mRNA per well.

10. Perform the final quantitative analysis. Input the data into an appropriate limiting dilution calculator software to determine the frequency of cells expressing spliced HIV-1 mRNA per million $CD4^+$ T-cells.

Note: We recommend the Extreme Limiting Dilution Analysis (ELDA) software, http://bioinf.wehi.edu.au/software/elda.[12] When this software is used for calculations, the input data will be as follows:

18000, 24, X_1.

9000, 24, X_2.

3000, 24, X_3.

1000, 24, X_4.

Where the first column is reserved for the dilution (cells per well), the second column for the number of replicates per dilution, and the last column (X_1, X_2 etc.) for the number of positive wells per dilution factor (Figure 4).

Note: the ELDA software returns a maximum likelihood estimate and 95% confidence interval (lower limit, upper limit). Multiply the estimates and 95% confidence interval by 1×10^6 to get the frequency of cells expressing spliced HIV-1 mRNA / 1×10^6 $CD4^+$ T-cells (Figure 4). If all

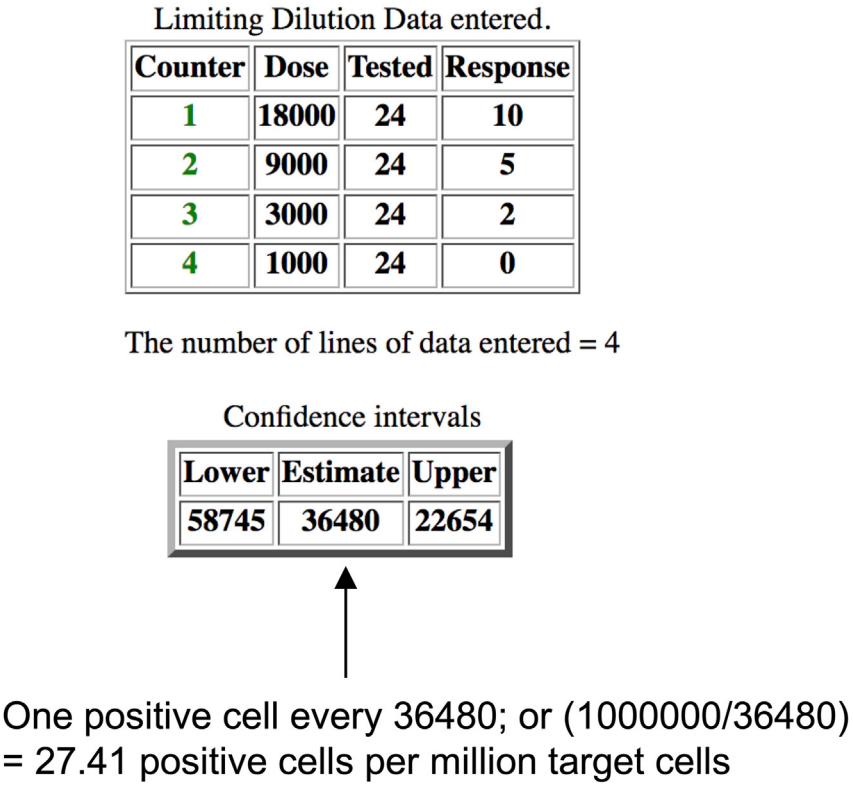

Figure 4. ELDA calculator input for quantitative TILDA analysis

The final quantitative analysis of TILDA data is performed with an appropriate limiting dilution calculator software. The Figure depicts an example of input and output data using the recommended ELDA software (http://bioinf.wehi.edu.au/software/elda.5) to determine the frequency of cells expressing spliced HIV-1 mRNA transcripts per million $CD4^+$ T cells.

wells are positive (extremely large reservoir) repeat the assay increasing the dilution factor. If all wells are negative (very small reservoir), repeat the assay with more replicate plates.

Day 3

Staining of dead cells with 7-AAD/Annexin V

Timing: 30 min

Part of the cells isolated on DAY1 were used for TILDA on DAY2. The remaining cells (i.e., unstimulated, activated with PMA/ionomycin, treated with the experimental drug/s) will now be used for viability sorting, followed by HIV-1 integrated DNA quantification in live sorted cells (to assess the "kill" phase of the therapeutic approach).

Since in DAY1 at least 2 × 10^6 cells were plated for each condition, and since TILDA requires less than 1 × 10^6 cells, there will be at least 1 × 10^6 cells available for the following steps.

11. Wash cells with cold PBS and resuspend them at a concentration of 1 × 10^6/mL in 1× Annexin Binding Buffer.
12. Add 5 μL of 7-AAD and 5 μL of Annexin V solution (provided in the kit) per 100 μL of cell suspension.
13. Gently vortex cells and incubate for 15 min at 15°C–25°C in the dark.

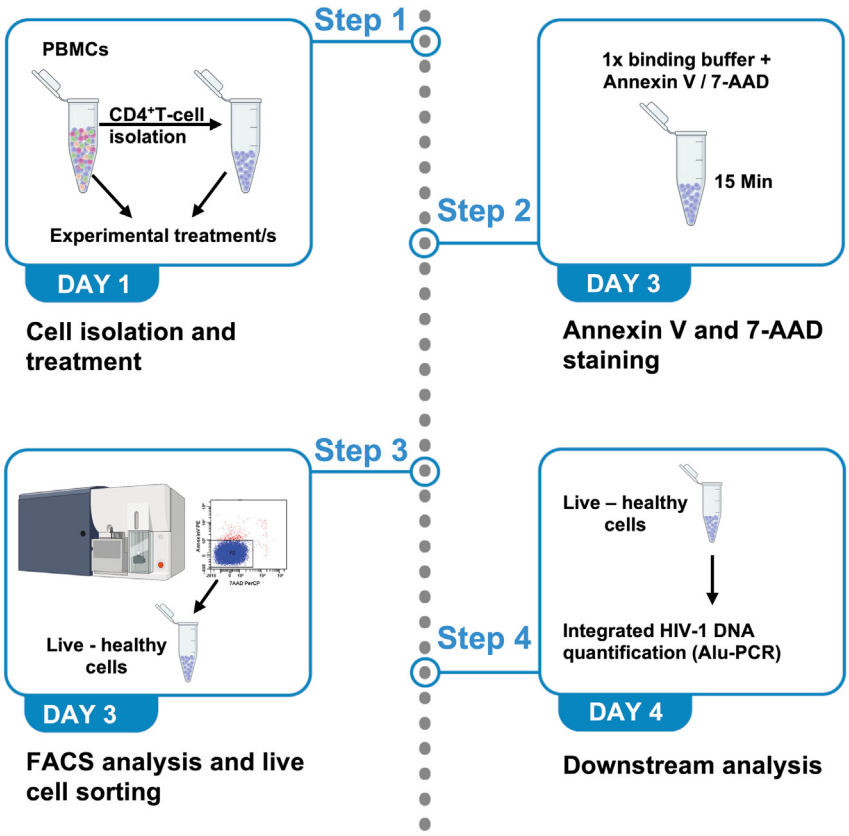

Figure 5. Schematic workflow of viable cell sorting using Annexin V/7-AAD staining

$CD4^+$ T-cells (or PBMCs) are isolated and treated with the investigational compound(s) of interest (step 1) (see also Figure 1). After typically 48 h, cells that were not used for TILDA are stained with Annexin V/7-AAD in binding buffer (step 2) and the population double negative for these markers is separated through sorting (step 3). Sorted cells are then used for downstream Alu-PCR analysis to quantify the amount of integrated HIV-1 DNA (step 4).

14. Pellet cells by centrifugation at 250 × *g* for 10 min and resuspend them at a concentration of 1 × 10^6/mL in 1× Binding Buffer. Cells are now ready for sorting and viable cell collection.

    Optional: a positive cell death control can be prepared by treating control cells isolated from healthy donors or surplus cells isolated from PLWH with compounds of known cytotoxicity (e.g., H_2O_2, doxorubicin, etoposide), or by incubating them at 60°C for 10 min. Results can vary and therefore a protocol to prepare a positive apoptosis control should be validated in advance.

    Note: the untreated population is used to define the basal level of apoptotic/dead cells to estimate the effect of experimental compound(s).

Sorting of viable cells

Timing: 15 min per sample

In this step (summarized in Figure 5) cells are analyzed by FACS and gated based on their physical parameters and negativity/positivity for Annexin V and 7-AAD. Viable cells (i.e., double negative for Annexin V and 7-AAD) are then sorted.

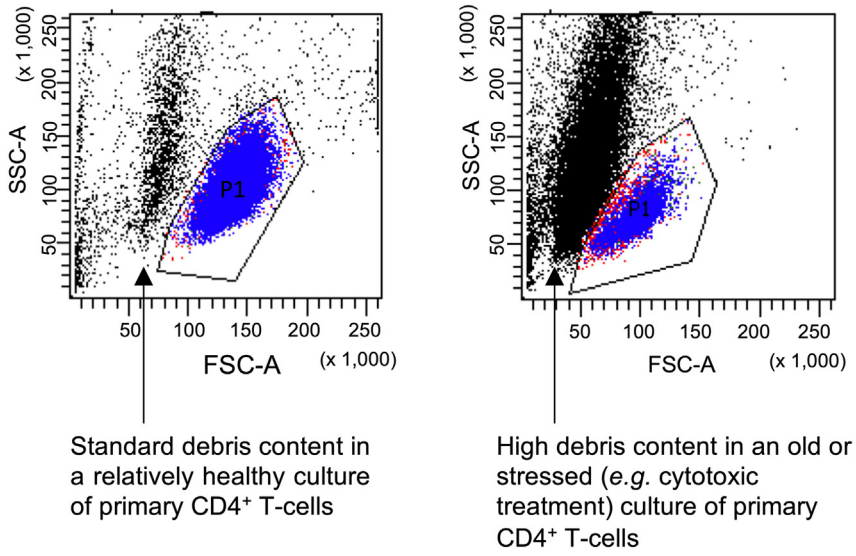

Figure 6. Representative gating strategy for debris exclusion in flow cytometric analysis

Exclusion of apoptotic bodies (debris) is based on the physical parameters visualized and gated on the forward (FSC) and side (SSC) scatter axes. The Figure depicts two examples of debris content/gating in $CD4^+$ T-cells: low amounts of debris in a mostly viable population (left panel) and high amounts of debris in a stressed/dying population (right panel).

15. Gate live cells based on forward scatter (FSC) and side scatter (SSC) parameters excluding the debris localized in the low FSC area of the FACS plot (Figure 6).
16. Analyze and sort healthy/live cells.
    a. Use the gating in step 14 to further gate live cells based on Annexin V/7-AAD expression, as shown in Figure 7.
    b. Sort cells that are negative for both Annexin V and 7-AAD fluorescence to obtain a live population for downstream HIV-1 DNA quantification.

Note: each cell culture includes apoptotic and dead cells which produce apoptotic bodies, characterized by a size that is a fraction of the total cell volumes (Figure 6). These bodies are normally distributed along the SSC axis but have low FSC values as compared to live cells. As this debris can contain both proteins and nucleic acids, it should not be gated, because it can lead to an overestimation of the HIV-1 DNA quantification. Therefore, only the events located outside this debris region should be gated and analyzed (Figure 6). As untreated fresh cells are normally poor in debris, they can be used to setup the appropriate gating strategy that can be then applied to all samples.

Optional: the population of cells double negative for Annexin V/7-AAD (healthy/live cells) can be analyzed and sorted for other relevant markers (e.g., CD4). Although this control could be useful in some studies, it could be biased in other cases, such as when the experimental treatment alters the expression or fluorescence of the marker analyzed (e.g., CD4).

Day 3–4

Lysate preparation for Alu-PCR

Timing: 1 day

In this step previously sorted, viable cells are lysed for downstream Alu-PCR analysis (Figure 8).

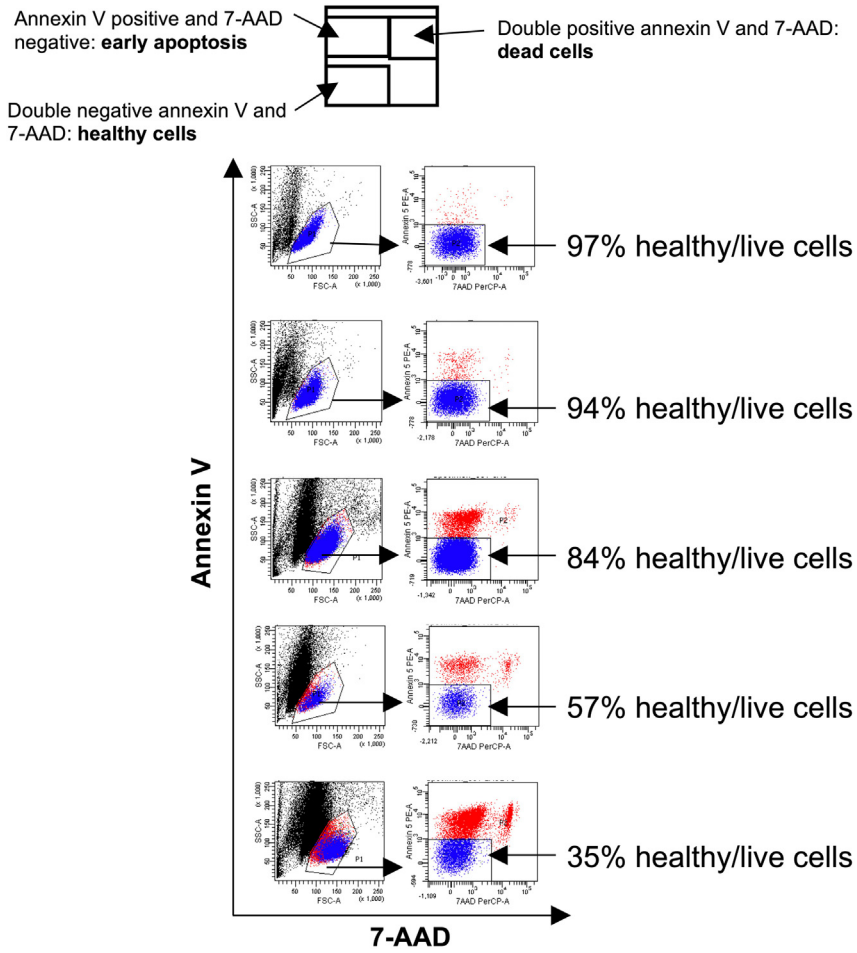

Figure 7. Representative outcomes of Annexin V/7-AAD analysis/sorting

The Figure depicts the gating profiles and expected percentages of viable $CD4^+$ T-cells (i.e., negative for both Annexin V and 7-AAD fluorescence) that can be sorted in typical experimental scenarios.

17. Wash and resuspend cells in PBS and count them.
18. Pellet cells and add pK solution according to the following formula: (number of cells/30,000) × 15 = X µL of pK solution (Figure 9). For example, 60,000 cells will be resuspended in 30 µL of pK solution (60,000/30,000 × 15 = 30 µL).
19. Transfer the vial with cells in pK buffer in a thermos-shaker and set 55°C for 16–24 h with gentle shaking.
20. The day after, transfer the vials in a 95°C thermos block for 5 min. The samples are now ready for use in Alu-PCR.

Note: The pK solution may be frozen multiple times with no efficiency loss.

Note: For samples with less than 30,000 cells, use 15 µL of pK solution.

Pause point: after step 19 the samples can either be used immediately or can be stored at −80°C for up to one week with no signal loss.

Day 4

The whole Alu-PCR assay procedure is summarized in Figure 8.

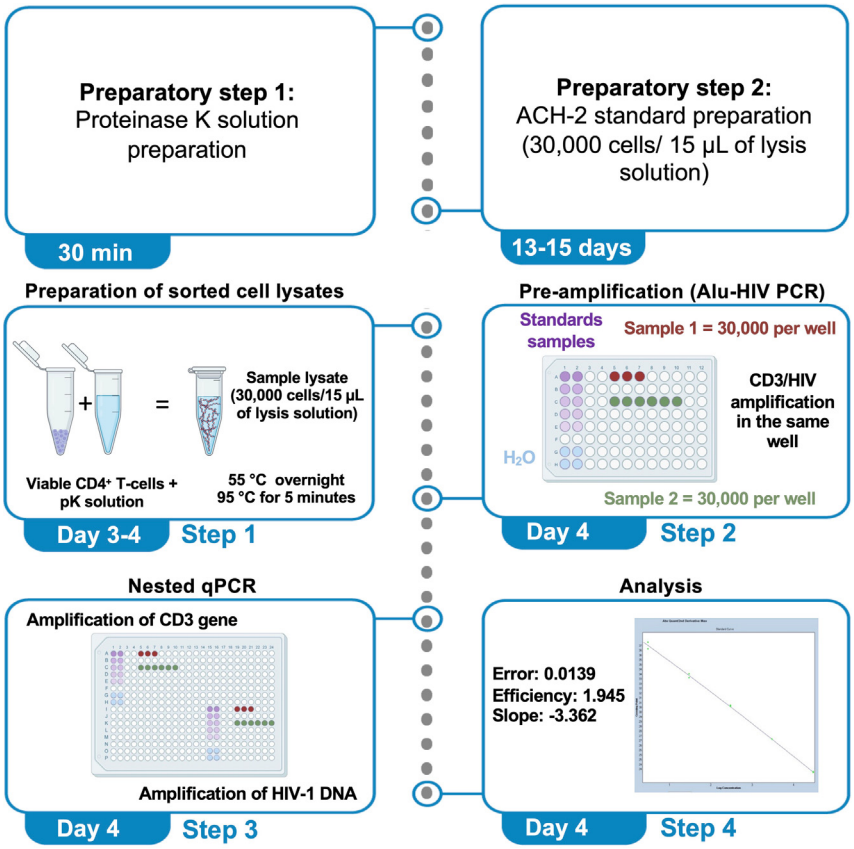

Figure 8. Schematic workflow of the Alu-PCR assay

The Alu-PCR protocol is used to quantify integrated HIV-1 DNA and, when applied downstream to viable cell sorting (Figures 5, 6, and 7), can be used to estimate the efficacy of the "kill" phase of the therapeutic strategy (i.e., elimination of infected cells). Following two preparatory steps (proteinase K solution preparation and standard curve preparation), the specific protocol includes four steps. Initially, previously isolated and sorted target cells (see Figures 1, 5, 6, and 7) are lysed in proteinase K buffer (step 1). This is followed by a pre-amplification PCR of the standard curve lysates and of the experimental lysates (Alu-HIV PCR, step 2). The pre-amplificates are then used in a nested qPCR (step 3) with primers directed against the HIV-1 genome or the CD3 gene (housekeeper control). Finally, data are analyzed and the number of integrated DNA copies per million target cells is calculated.

In this step the standard curve serial dilutions will be prepared. Afterward, the standard curve and experimental samples will be loaded in a pre-amplification plate to carry out the first PCR reaction (Alu-HIV PCR).

Alu-PCR pre-amplification ("kill" phase)

Timing: 5 h

21. Prepare the pre-amplification master mix as shown in Table 5 below:
22. Prepare serial dilutions for the standard curve (range: 3–30,000 copies).
    a. Thaw at 15°C–25°C a 30 µL aliquot of ACH-2 standard (prepared as described in the "before you begin" paragraph, step 6). Label this as tube A.
    b. Label four empty microcentrifuge tubes as tube B to E and fill each with 90 µL H_2O.
    c. Transfer 10 µL from tube A to tube B and pulse vortex tube B five times.
    d. Changing tip each time, repeat the dilution/vortex procedure sequentially (i.e., transfer 10 µL from tube B to C, 10 µL from C to D and 10 µL from D to E).

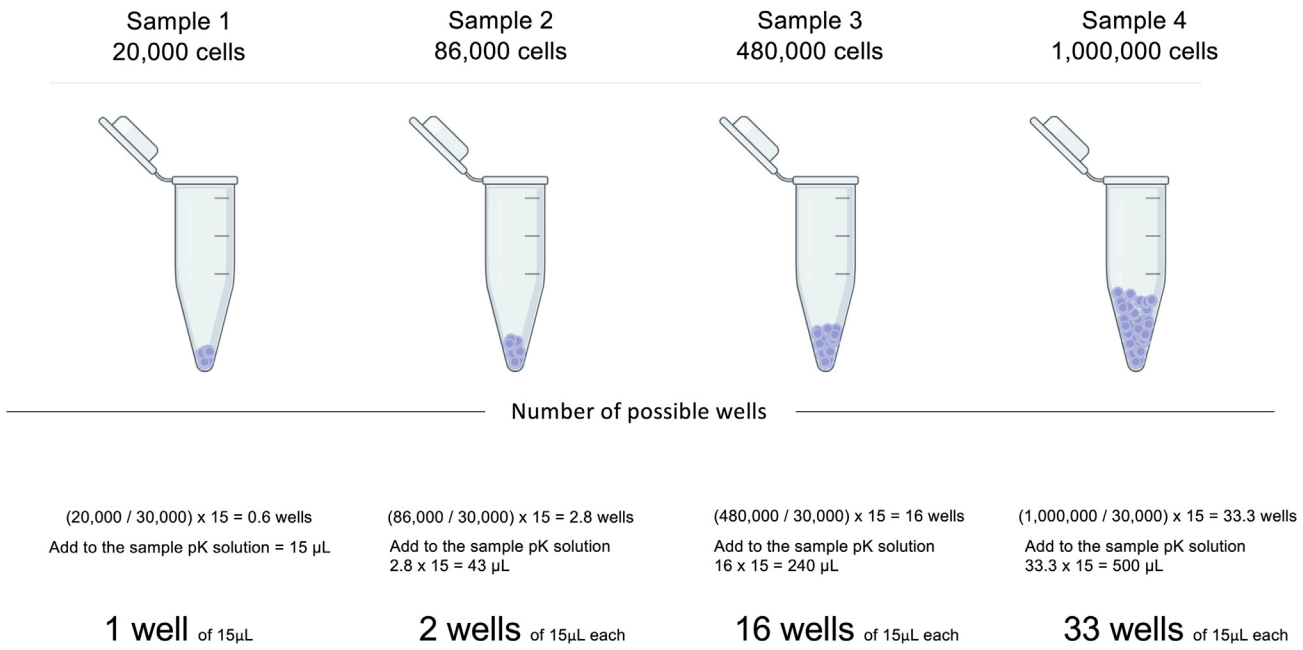

Figure 9. Calculation examples of the number of wells required for different cell inputs in Alu-PCR

Each sample consists of 30,000 cells per well diluted in 15 µL of lysis (pK) buffer. Even if less than 30,000 cells should be available (e.g., sample 1) 15 µL of lysis (pK) buffer should be used.

e. Keep the 5 standard tubes on ice until use.

23. Load the pre-amplification plate.
    a. Put the pre-amplification plate on a PCR cooler or on ice to avoid nonspecific reactions.
    b. Add 35 µL of master mix to each well.
    c. Load ten wells with 15 µL of each standard dilution (from A to E) in duplicate (as shown in Figure 10).
    d. Likewise, load each experimental sample using 15 µL of cell lysate per well (prepared as described in sections 17–20 above).
    e. Leave at least four wells loaded only with master mix and 15 µL H_2O to serve as negative controls.
    f. Spin the plate for 30 s at max speed and load the plate in a PCR instrument.

Note: We suggest testing experimental samples in triplicate or quadruplicate, when possible (i.e., prepare a lysate of at least 100,000 cells per sample) (Figure 10).

Table 5. Calculations for master mix preparation of the pre-amplification plate of Alu-PCR

Reagent	Final concentration (in 50 µL)	Amount (µL)
H_2O	N.A.	23.65
Taq Buffer 10×	1×	5
$MgCl_2$ 50 mM	3 mM	3
dNTP mix 10 mM	0.3 mM	1.5
primer HCD3OUT5′ 50 µM	300 nM	0.3
primer HCD3OUT3′ 50 µM	300 nM	0.3
primer ULF1 50 µM	150 nM	0.15
primer Alu1 50 µM	300 nM	0.3
primer Alu2 50 µM	300 nM	0.3
Taq polymerase (5 U/µL)	0.05 U/µL	0.5
Total		**35**

Amounts are calculated for one well.

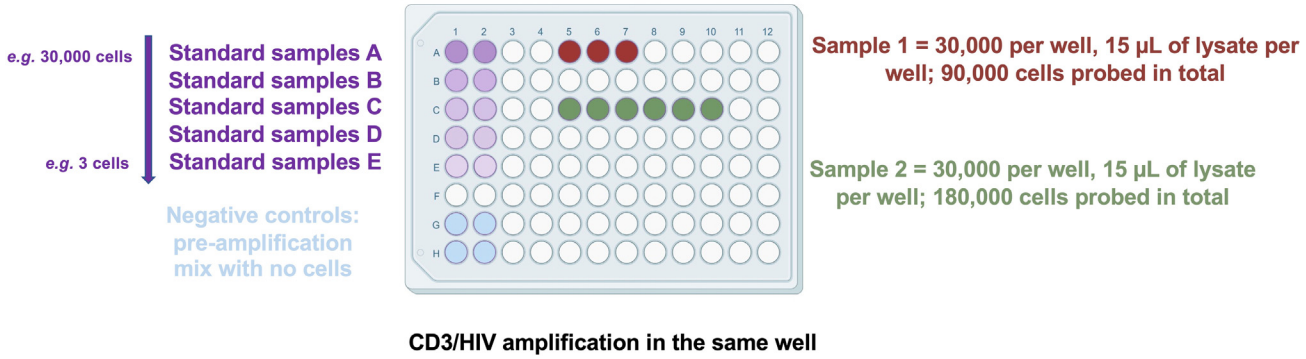

Figure 10. Schematic depiction of optimal sample/control distribution in the pre-amplification plate (Alu-HIV plate)

The pre-amplification PCR plate includes serial dilutions of the standard curve (range: 3–30,000 copies, in purple), the negative control (H_2O, light blue) and the experimental samples (typically 30,000 cells per sample) using three (red) or six (green) replicates, depending on the amount of starting material available.

Note: The pre-amplification plate must be kept on ice when not used immediately. Moreover, the plate should be prepared and stored in an amplicon-free room, generally designed as pre-PCR room.

24. Perform the first round of PCR amplification.
    a. Set the lid of the PCR instrument at 110°C to avoid sample evaporation.
    b. Set the parameters of the PCR as shown in Table 6 and start the PCR reaction.

⚠ CRITICAL: The pre-amplification is the most important technical step of the Alu-PCR protocol. In this step Alu-LTR amplicons of different size will be generated (up to 10 kb in length). Long denaturation and extension times are required to ensure the generation of these amplicons. In our experience, instruments with low heating and cooling rates perform better, while fast cycling instruments should be avoided. We generally use an Eppendorf Mastercycler Pro instrument with heating and cooling rates of 4°C and 3°C per second, respectively. In addition, we have also tested the ABI Geneamp 9700 and Biometra instruments with positive results. When setting-up the assay for the first time, we suggest performing the tests while running the ACH-2 standards with different PCR instruments and cycles (typically between 8 and 12 cycles). The amplification conditions may be considered satisfactory when all five standard curve dilutions are positive, and the slope of the curve meets the criteria outlined in the "Expected outcome" paragraph.

Preparation, run and analysis of the nested real-time PCR ("kill" phase)

Timing: 4 h

In this step the pre-amplificates prepared as described in the previous paragraph will be incubated with two qPCR reaction mixes, one for the detection of the CD3 gene (housekeeper) and one for the

Table 6. Parameters of first round Alu-PCR amplification (Alu-HIV PCR)

Steps	Temperature	Time	Cycles
Denaturation	95°C	8 min	1
Denaturation	95°C	1 min	8–12 cycles
Annealing	55°C	40 s	
Extension	72°C	1 min	
Extension	72°C	15 min	1
Hold	4°C	∞	

Table 7. Calculations for preparing the two master mixes for the nested PCR plate

Reagent		Final concentration (in 20 µL)	Amount (µL)
CD3 mix	HIV mix		
Faststart essential DNA probes master 2×		1×	10
Primer CD3in (100 µM)	primer LambdaT (100 µM)	1.25 µM	0.25
Primer CD3in (100 µM)	Primer UR2 (100 µM)	1.25 µM	0.25
Probe CD3 (10 µM)	Probe HIV (10 µM)	250 nM	0.5
ddH_2O		N.A.	2.6
Total			**13.6**

Amounts are calculated for one well.

detection of integrated HIV-1 DNA. The CT values obtained will then be used to quantify the amount of integrated HIV-1 DNA in each cell lysate examined.

25. Prepare the two nested real-time PCR mixes (i.e., for CD3 and HIV-1 DNA) as described in Table 7 below.

Note: if not used immediately the mixes must be stored at 4°C.

26. Load in triplicate the nested PCR plate with 13.6 µL of mix per well keeping separate wells containing either the CD3 mix or the HIV mix.
27. Recover the Alu-HIV plate at the end of the first PCR run (described in Table 6) and gently remove the cover film.
28. Transfer 10 µL from each well of the Alu-HIV plate in a new PCR plate.
29. Add 90 µL of H_2O per well and gently mix with a multichannel pipette.
30. Transfer 6.4 µL of the diluted pre-amplification product (i.e., Alu-HIV product) in the nested PCR plate (i.e., both in the wells with CD3 mix and in the wells with HIV mix) (Figure 11).

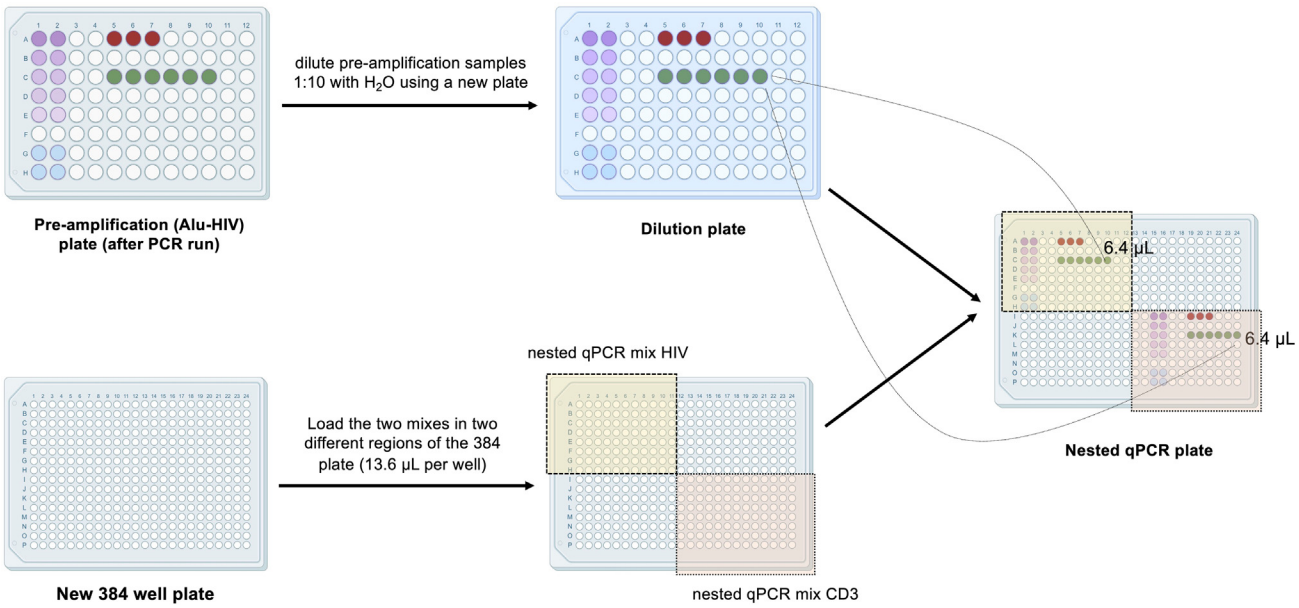

Figure 11. Schematic depiction of optimal sample/control distribution in the nested qPCR plate for integrated HIV-1 DNA quantification

Depicted are the steps required for sample/control and master mix preparation which are then combined in the final nested PCR plate. For the master mix, the two reaction mixes for CD3 and HIV are prepared and loaded separately in the nested qPCR plate. For sample/control preparation, the pre-amplification (Alu-HIV) plate is diluted 1:10 in the dilution plate and 6.4 µL of each well from the dilution plate are loaded in the nested qPCR plate. For the color code of the standards/samples, see Figure 10.

Table 8. Conditions of nested PCR reaction for integrated HIV-1 DNA quantification

Steps	Temperature	Time	Cycles
Pre-incubation	95°C	10 min	1
Denaturation	95°C	10 s	50 cycles
Annealing and Extension	60°C	30 s	
Final Extension	72°C	1 s	
Cooling	40°C	30 s	
Hold	25°C	∞	

Note: to increase the throughput of the technique, and if a compatible instrument should be available, it is recommended to load the nested qPCR in a 384 well-plate, as the nested qPCR will contain twice the number of samples (due to the two different mixes) as compared to the Alu-HIV plate (Figure 11).

31. Seal the nested PCR plate with a real time PCR grade sealing foil and spin it at 700 × *g* (or max speed) for 30 s.
32. Transfer the plate in the real-time PCR instrument and run the qPCR using the program shown in Table 8 below.

Note: in case promising pharmacological "shock and kill" candidates are identified, it is strongly recommended to repeat the assay with cells from multiple donors and in increased amounts to improve the precision of the technique.

33. Analyze the data.
    a. At the end of the nested qPCR reaction, quantify the CD3 amount per well and the HIV-1 copy number using the absolute quantification method according to the instructions of the qPCR machine used.
    b. Use the CD3 and HIV-1 DNA quantifications to calculate the ratio (HIV / CD3) × 10^6 and obtain the copy number of HIV per million cells, as shown in Table 9.

Note: the absolute quantification method can be easily calculated by modern qPCR machines performing a second derivative analysis (although alternative methods, such as the fit points analysis, can be used as well). This leads to a count of CD3 and HIV-1 DNA molecules for each well, based on the respective CD3 and HIV standard curves.

EXPECTED OUTCOMES

The workflow has confirmed *ex vivo*[1] the effects of a combination of two drugs (auranofin and buthionine sulfoximine) able to abate the viral reservoir in a monkey AIDS model.[13] Interestingly, also the

Table 9. Representative analysis procedure for integrated HIV-1 DNA quantification

Steps	Procedure	
Step 1	Unknown sample = e.g.,., 90,000 cells; 3 wells (30,000 cells per well, 15 µL cell lysate per well).	
Step 2	Preamplification and nested qPCR for CD3 and HIV quantification.	
Step 3	Calculate standard curves for CD3 and HIV.	
Step 4 (e.g.,)	Extrapolate the values for CD3 and HIV for each well.	
	CD3 copies	HIV copies
	32,000	25
	31,100	19
	31,800	25
Step 5	Sum of CD3 copies	Sum of HIV copies
	94,900	69
Step 6	Ratio calculation (copies of HIV DNA per 1,000,000 cells).	
	(69/94,900) × 1,000,000 = 727 copies per million cells.	

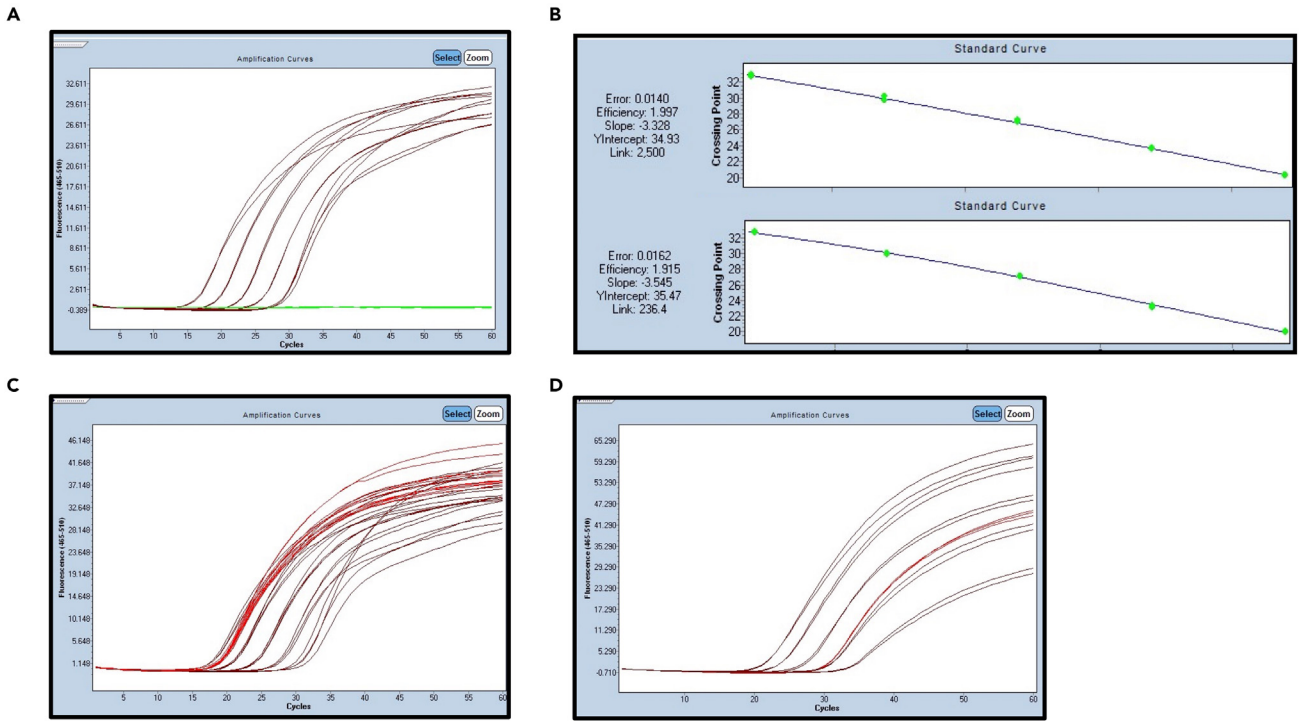

Figure 12. Representative examples of the amplification results of the nested qPCR assay for integrated HIV-1 DNA quantification

(A and B) Examples of ACH-2-derived standard curves for CD3, in duplicate (A), and of their calculated slope and efficiency (B).

(C and D) Examples of CD3 (C) and HIV (D) curves generated by loading experimental samples (triplicate, in red) and ACH-2-derived standards (quadruplicate, in brown).

results obtained with the histone deacetylase inhibitor vorinostat[1] were in line with previous findings showing that this drug might exert a reactivating ("shock") effect[14,15] but does not reduce the viral reservoir in patients.[16] Therefore, drugs/drug combinations identified by this method and active at concentrations therapeutically achievable in plasma are likely to exert an activity *in vivo*. This is especially relevant for repurposed drugs, with known pharmacokinetics, while experiments with new molecules will likely be more dependent on multiple *in vivo* validations to identify a safe and effective dosing interval.

In TILDA, after stimulation with PMA/ionomycin, $CD4^+$ T cells (or PBMCs) should be richer in spliced HIV-1 mRNA transcripts when compared to unstimulated cells. Likewise, treatment with drugs effective in reactivating ("shock") the functional reservoir should result in TILDA values higher than in the unstimulated control.

In the Alu-PCR assay the standard curves for CD3 and HIV (e.g., Figure 12) should ideally have: a slope of -3.3 (although an interval between -3.1 and -3.7 can be acceptable), an error < 0.03, and an efficiency ~ 2 [calculated as efficiency (E) = $10^{-1/slope}$, e.g., if slope = -3.3 then E = 2]. After each experimental well is automatically quantified for CD3 and HIV-1, the calculated abundancy values should be comprised in the standard limits (>3 and <30,000 copies).

The comparison between the untreated and the treated samples by Alu-PCR is necessary to assess the "kill" effect of a candidate drug. In theory, cells treated with an effective "kill" compound should have less HIV-1 DNA copies when compared to the untreated sample.

Overall, a "shock and kill" drug or drug combination will result in higher TILDA and lower Alu-PCR signals as compared to the untreated control.

LIMITATIONS

The quality and number of input cells is a crucial parameter. Although TILDA can be performed with lower amounts of cells or with a lower number of replicates, this can impact on the precision of the assay.

The Alu-PCR protocol has been validated in multiple studies, but a proper setup before the experiment is essential, in line with the requirements of any absolute quantification protocol. In general, only experiments where the standard curves overlap with the parameters described in the expected outcomes section can be regarded as reliable.

Although Alu elements are dispersed throughout the cellular genome,[17] if the integrated HIV-1 DNA should not reside at a distance comprised within typical amplicon lengths, the viral DNA copy will not be detected. Moreover, the Alu-PCR technique cannot distinguish between full length and defective genomes, for the analysis of which sequencing techniques are necessary.[18]

The sorting of viable cells removes dying/dead cells that could skew the Alu-PCR readout but does not distinguish between infected and uninfected cells. Therefore, although the assay offers a quantitative estimate of the "kill" phase of the therapeutic strategy, the tolerability of the compound(s) tested may need to be further evaluated through dedicated assays.

TROUBLESHOOTING

Problem 1

No positive signals after the nested qPCR step in TILDA (step 9).

Potential solution

- Repeat the experiment double checking that PCR mixes and amplification protocols have been correctly prepared and followed.
- Repeat the experiment testing a known reactivating stimulus in a cell line stably infected with HIV-1 (e.g., ACH-2 or OM10). This will serve as positive control that should generate positive signals in every well.

Problem 2

All wells are positive after the nested qPCR step in TILDA (step 9).

Potential solution

When testing patient-derived cells this is generally due to a contamination by a previous PCR amplification run.

- Clean pipettes and biosafety cabinets used during TILDA with a RNase/DNase solution (many commercial formulations are available).
- Replace one by one all the reagents used during the PCR steps until the problem is solved.

Problem 3

The slope of the Alu-PCR standard curves is outside the expected values (i.e., not -3.3 or at least > -3.1 and < -3.7) or some standard curves are not generated (step 33 and expected outcomes).

Potential solution

- Prepare a fresh culture of ACH-2 cells and standards. The 30,000 cells required for the standard should be manually counted by at least 2 different operators, multiple times, to establish a robust and reliable cell number.
- Prepare a fresh pK solution.

- Modify the number of cycles in the pre-amplification (Alu-HIV) PCR, using between 8 to 12 cycles.
- Try a different PCR instrument.

Problem 4

No signal for both CD3 and HIV amplification after Alu-PCR (step 33).

Potential solution

- Prepare a new stock of proteinase K solution and double check whether the thermos block is correctly set at 55°C during digestion and at 95°C during proteinase K disruption (see the "preparation of proteinase k (pK) solution" section).
- Test a new Taq polymerase aliquot (step 21).
- Double check the primer dilutions (steps 21, 25).

Problem 5

All cells are AnnexinV/7-AAD positive (step 16b).

Potential solution

- Perform the AnnexinV/7AAD analysis on a validated, viable cell line. If also in this case all cells are positive, prepare fresh reagents (RPMI, PBS, new AnnexinV/7-AAD kit).
- If the cell line results after AnnexinV/7-AAD staining are negative, check culture conditions of primary cells (cell concentration, incubator, RPMI medium).
- If the cell line results after AnnexinV/7-AAD staining are positive for the majority of cells, then the drug(s) at the tested concentrations have an unacceptable toxicity profile, and the treatment strategy will need to be revised.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Iart Luca Shytaj (luca.shytaj@bristol.ac.uk).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new datasets.

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AUTHOR CONTRIBUTIONS

Conceived the work, F.A.P., A.S., I.L.S.; Wrote the manuscript, F.A.P., I.L.S.; Drafted figures, F.A.P. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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