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An Optimized Tat/Rev Induced Limiting Dilution Assay for the Characterization of HIV-1 Latent Reservoirs

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Abstract

The administration of antiretroviral therapy (ART) leads to a rapid reduction in plasma viral load in HIV-1 seropositive subjects. However, when ART is suspended, the virus rebounds due to the presence of a latent viral reservoir. Several techniques have been developed to characterize this latent viral reservoir. Of the various assay formats available presently, the Tat/Rev induced limiting dilution assay (TILDA) offers the most robust and technically simple assay strategy. The TILDA formats reported thus far are limited by being selective to one or a few HIV-1 genetic subtypes, thus, restricting them from a broader level application. The novel TILDA, labelled as U-TILDA ('U' for universal), can detect all the major genetic subtypes of HIV-1 unbiasedly, and with comparable sensitivity of detection. U-TILDA is well suited to characterize the latent reservoirs of HIV-1 and aid in the formulation of cure strategies.

Keywords: TILDA, HIV-1 latency, Latent reservoir, HIV-1, Transcriptional silence

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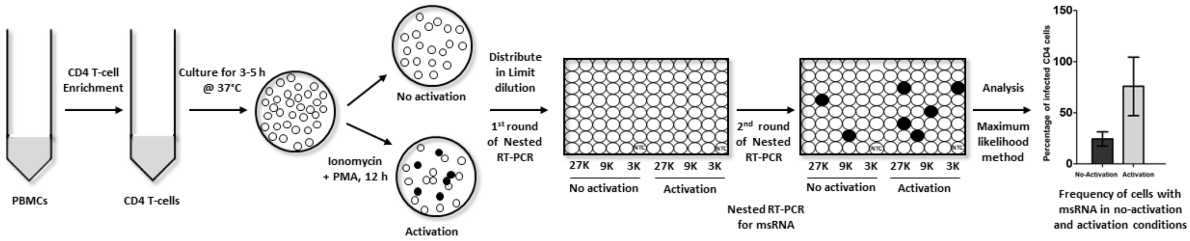
Graphical abstract:



Background

Latent reservoir (LR) poses a significant obstacle to HIV-1 disease management. Despite successful antiretroviral therapy (ART), low-level viral replication persists, attributed to the existence of a transcriptionally silent virus (Chun *et al*., 1995, 1997; Finzi *et al*., 1997; Siliciano *et al*., 2003). Precise quantitation of the size of this latent reservoir before and after the medical intervention is critical for 'cure' or therapeutic research.

A variety of assay formats have been developed to characterize HIV-1 latent reservoirs (Falcinelli *et al*., 2019). These assays differ from one another in terms of the viral product they measure, complexity, and sensitivity, among other attributes. DNA PCR-based assays offer the technically simplest experimental format. However, such assays quantify total proviral DNA, regardless of replication competence of the integrated viruses, thus, overestimating the overall viral reservoir size (Massanella and Richman, 2016). The highly popular quantitative viral outgrowth assay (QVOA) measures the number of infectious viral units per million CD4 T-cells (Siliciano and Siliciano, 2005). The QVOA, considered to be the gold standard technique, suffers from several limitations, including the complex experimental design, and the need for a large quantity of blood (120–180 mL). Additionally, since only a fraction of replication-competent proviruses is activated under any experimental condition, QVOA tends to underestimate the size of the viral reservoir.

Among a horde of assays available to characterize HIV-1 latent reservoirs, a strategy that aims to detect the frequency of cells producing viral transcripts offers the most powerful platform. The Tat/rev induced limiting dilution assay (TILDA) can measure cells producing multiply-spliced viral transcripts following cell activation (Procopio *et al*., 2015). The TILDA format offers a realistic approximation of the size of the replication-competent latent reservoir, as it detects HIV-1 transcripts, not proviral DNA. Importantly, TILDA exploits the power of PCR amplification, thus offering a highly sensitive experimental format. Additionally, unlike QVOA, TILDA requires a significantly smaller volume of a clinical sample (15–20 mL). Despite these considerable technical merits, TILDA is highly susceptible to the genetic variation of several HIV-1 viral subtypes. Therefore, the several TILDA platforms reported thus far are limited by the ability to target a specific HIV-1 genetic family, or even only a proportion of viral strains of a subtype (Bertoldi *et al*., 2020; Leyre *et al*., 2020). We recently reported a novel TILDA assay format labelled as U-TILDA ('U' for universal), that can target all the major genetic subtypes of HIV-1 (A, B, C, D, and AE subtypes) with high sensitivity and comparable diagnostic efficiency (Mehta *et al*., 2021). In contrast to subtype-specific TILDA formats, in U-TILDA, the primer pairs and the probe target the highly conserved exons of HIV-1, thus significantly broadening the breadth of detection of the assay.

Here, we describe the detailed protocol of U-TILDA using stored PBMC of an HIV-1 seropositive subject. The CD4 T-cells, negatively enriched using paramagnetic beads, and activated with PMA and ionomycin for 12 h, or left without activation for control, were serially diluted. One step qRT-PCR was performed in sixteen replica wells for each cell dilution. The viral transcripts were converted into cDNA using HIV-1-specific RT primers, without RNA purification. This was followed by the first-round of PCR amplification in the same reaction vial. Diluted PCR reaction content was then used as the template for the second-round of the nested-PCR, in a real-time PCR format. Finally, the Ct values of the replicate wells were converted to the frequency of transcription-competent viral events using a free web tool.

Materials and Reagents

A. Consumables and reagents

1. Conical centrifuge tubes, 50 mL (Thermo Fisher Scientific, catalog number: 14-432-22)
2. Conical centrifuge tubes, 15 mL (Thermo Fisher Scientific, catalog number: 14-959-53A)
3. Round-bottom polystyrene tubes, 5 mL (Thermo Fisher Scientific, catalog number: 14-959-1A)
4. Microcentrifuge tubes; 0.5, 1.5, and 2 mL (Tarsons, catalog numbers: 500000, 500010, and 500020 respectively)
5. PCR plate (Bio-Rad Laboratories, catalog number: HSP9601)
6. RPMI 1640 medium (HiMedia Laboratories, catalog number: AL162S)
7. Fetal bovine serum (FBS, Heat-inactivated) (Thermo Fisher Scientific, catalog number: 10082147)
8. Penicillin G (Sigma-Aldrich, catalog number: P3032)
9. L-glutamine (Sigma-Aldrich, catalog number: G8540)
10. Streptomycin (Sigma-Aldrich, catalog number: S9137)
11. EasySep Human $CD4^+$ T cell isolation kit (Stemcell Technologies Inc., catalog number:19052)
12. Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, catalog number: P1585)
13. Ionomycin (Sigma-Aldrich, catalog number: I0634)
14. SuperScript III Platinum One-Step qRT-PCR Kit (Invitrogen, catalog number: 11732020)
15. My*Taq*™ DNA polymerase (Bioline, catalog number: BIO-21105)
16. PCR sealant (Bio-Rad Laboratories, catalog number: MSB1001)
17. FITC Mouse Anti-Human CD4 antibody (BD Biosciences, catalog number: 566802)
18. FITC Mouse IgG1, κ Isotype Control (BD Biosciences, catalog number: 349041)
19. APC Mouse Anti-Human CD3 antibody (BD Biosciences, catalog number: 555342)
20. APC Mouse IgG2a, κ Isotype Control (BD Biosciences, catalog number: 550882)
21. Live/Dead Fixable Far Red Dead Cell Stain Kit, for 633 or 635 nm excitation (Thermo Fisher Scientific, catalog number: L10120)
22. Peripheral blood mononuclear cells (PBMC)
23. Revival media (see Recipes)
24. Enrichment media (see Recipes)
25. Staining buffer (see Recipes)
26. RPMI medium supplemented with 10% FBS (see Recipes)
27. Phosphate-buffered saline (PBS) solution (see Recipes)
28. Tris-EDTA (TE) buffer (see Recipes)
29. 40 µg/mL PMA (see Recipes)
30. 4 µg/mL Ionomycin (see Recipes)

B. Primers and probe (Table 1)

Table1. Details of the primers and probe used in PCR and cDNA synthesis

Oligonucleotides	Identity	Coordinates (HXB2)	Sequence (5'-3')
Primers	N2830 (OFP)	514-540	CTGCTTAAGCCTCAATAAAGCTTGCCT
	N2831 (ORP)	8510-8539	CTCAATCGGTGGTAGCTGAAGAGGCACAGG
	N2832 (IFP)	577-603	GACTCTGGTAACTAGAGATCCCTCAGA
	N2833 (IRP)	8419-8448	TGTCTTGCTCTCCACCTTCTTCTTCGATTC
Probe	N2842	683-709	(FAM)-CTCTCGACGCAGGACTCGGCTTGCTGA-(BHQ-1)
RT primers	N4101	9031-9046	TTTTTTTTTTTTTTCAGAGCACTC
	N4102	9032-9046	TTTTTTTTTTTTTCAGAGCACTCAAG
	N4103	9033-9046	TTTTTTTTTTTTTTCAGAGCACTCAAGG

Notes:
a. OFP and ORP: Outer Forward and Reverse Primers, IFP and IRP: Inner Forward and Reverse Primers, RT primers: Reverse Transcription primers.
b. The reverse primer sequences are presented in the reverse complement.
c. The primers were synthesized by Sigma-Aldrich.

Equipment

1. Liquid Nitrogen container (IOCL Cryocan)
2. 4°C refrigerator (Samsung)
3. –80°C freezer (Thermo Scientific)
4. CO_2 incubator (NuAire)
5. Water bath (BS Enterprises)
6. CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories)
7. Laminar flow hood (NV-Equipes)
8. Inverted microscope (Leica DM IRB)
9. Flow cytometer (BD FACSAria™ III)
10. Hemocytometer (TECH- LABCARE)

Software

1. FCS 6 Express™ Flow Cytometry (De Novo™ Software)
2. GraphPad Prism 5 (GraphPad Software, Inc.)
3. Extreme Limiting Dilution Analysis (ELDA) (Walter + Eliza Hall Bioinformatics, Institute of Medical Research). A free web tool available at https://bioinf.wehi.edu.au/software/elda/

Procedure

A. Revival of PBMC

1. Remove the required number of vials containing stored PBMC from the liquid nitrogen container and thaw the cells immediately at 37°C using a water-bath.

Notes:
a. At least 2×10^6–3×10^6 enriched $CD4^+$ T-cells will be required for a single assay (see below).
b. The CD4 percentage in healthy people is typically 30–40% of total lymphocytes, but this could be significantly lower in ART-naïve HIV-1 infected people. Therefore, a single assay may require 30×10^6–50×10^6 stored PBMC.
c. Further, it could be essential to consider additional experimental parameters that may cause cell loss. For example, cell storage, revival, and activation may lead to the death of a small proportion of cells. Likewise, flow cytometry validation may require setting aside a small fraction of cells (see step 4 below).
d. When working with HIV-infected cells, the following fundamental precautions must be taken:
  i. All experiments must be performed in a Biosafety Level-3 laboratory (BSL-3) or in a 'BSL-2 Plus' laboratory (BSL-2+), which is a 'BSL-3 laboratory minus negative pressure'. Please note that a regular BSL-2 is not suitable for HIV-1 work.
  ii. Researchers must stringently follow all safety procedures mandated for a BSL-3 facility.
  iii. See the following reference for more information on the operational procedures (Duane, 2013).

2. In a culture hood, pool the PBMC in a 15-mL or 50-mL plastic tube containing the revival medium at room temperature (mix sample and revival medium volumes at a ratio of 1:3).

> *Note: Before thawing the PBMC, dispense the required volume of the revival medium in a plastic tube readily available in the culture hood.*

3. Determine the cell count using a hemocytometer.
4. Set aside an aliquot of 0.3×10^6 PBMC in a plastic vial, for the flow cytometry analysis.

> *Notes:*
> *a. These cells will represent the 'pre-enrichment' sample. A similar sample will be collected later (see step 9 below) following the $CD4^+$ T-cell enrichment, labelled as the 'post-enrichment' sample. A comparison of these two samples by flow cytometry will identify the fold-enrichment of the $CD4^+$ T-cells (see Procedure C below).*
> *b. Additionally, these cells will also be used to account for two additional controls for flow cytometry, comprising the 'No stain' and the 'Isotype' controls.*

B. Enrichment of $CD4^+$ T-cells

5. Centrifuge the plastic tube containing the PBMC pool at $750 \times g$ and room temperature for 10 min. Decant the supernatant, and gently loosen the cell pellet.
6. Resuspend the pellet of approximately 50×10^6 PBMC in 1 mL of the enrichment medium at room temperature and transfer the cells to 5-mL round-bottom polystyrene tubes.
7. Use the EasySep Human CD4 T-cell isolation kit to enrich the $CD4^+$ T-cells from PBMC, by following the manufacturer's instructions.
8. Collect the enriched $CD4^+$ T-cells in a fresh 15-mL plastic tube containing 2 mL of RPMI medium supplemented with 10% FBS, at room temperature.
9. Determine the cell count and keep aside an aliquot of 0.1×10^6 cells, for the flow cytometry analysis.

> *Notes:*
> *a. Label the tube as the 'post-enrichment' sample.*
> *b. Approximately, 2×10^6–3×10^6 enriched $CD4^+$ T-cells are expected at this stage, especially from a clinical sample.*

10. Spin the rest of the cells and resuspend the cell pellet in 2.0 mL of complete RPMI medium. Dispense 950 µL of the cell suspension into two wells each of a 24-well cluster plate and incubate at 37°C for 3–5 h. Label the wells as "No activation" and "Activation". One of the two cell populations will be activated, while the other will serve as 'No activation' control in U-TILDA.

> *Note: The incubation will allow the cells to recover, following revival.*

C. Validation of $CD4^+$ T-cell enrichment by flow cytometry

Two different samples have been saved above (steps 4 and 9) before and after the cell-enrichment procedure. Unless otherwise mentioned, all the steps below are performed at room temperature.

11. Centrifuge the tubes with cells from steps 4 and 9 at $750 \times g$ for 5 min. Aspirate the medium, loosen the cell pellets, resuspend the cells in 1 mL of PBS each (PBS should be at room temperature), and centrifuge at $750 \times g$ for 5 min.
12. Aspirate the supernatant, loosen the cell pellets, and resuspend the cells in 100 µL of PBS (PBS should be

at room temperature).

13. Divide the 'Pre-enrichment' cells into three vials by transferring 30 µL of the cell suspension to each vial. Label the vials as 'No stain' control, 'Isotype' control, and 'pre-enrichment' sample. Add 60 µL of PBS to each vial to make the total volume 90 µL. Likewise, suspend all the cells of the 'post-enrichment' sample in 90 µL of PBS.

Note: The two control vials will be common for the pre- and post-enrichment samples.

14. Add 5 µL each of mouse anti-human CD4 antibody-FITC conjugate and mouse anti-human CD3 antibody-APC conjugate to the 'pre-' and 'post-enrichment' vials. Similarly, add 5 µL each of mouse IgG1, κ Isotype antibody-FITC conjugate, and mouse IgG2a, κ Isotype antibody-APC conjugate to the 'Isotype control' vial.
15. Incubate the samples at 4°C in the dark for 45 min.
16. After the incubation, wash the cells twice with 500 µL of the staining buffer by centrifugation at 750 × *g* for 5 min, aspirate the staining buffer, and loosen the cell pellet.
17. Resuspend the cells in 100 µL of staining buffer.
18. Acquire the sample data using a flow cytometer.

Note: Ideally, the stained samples should be analyzed as soon as possible. If necessary, the samples may be stored for 3–4 h at 4°C.

19. First, identify the lymphocyte population by defining the FSC and SSC scatters, using the 'No stain' control (**Figure 1A**). Then, place a quadrant on the living cell population using the CD3-APC and CD4-FITC staining (**Figures 1B–D**). Using the defined gate, analyse the rest of the samples.

Notes:

a. It may be necessary to determine the live cell population by adding a live-dead staining step to the protocol. A small proportion of PBMC may be dead after reviving the cells. The live-cell proportion must be 70% or higher.

b. Cells are subjected to the live-dead staining protocol before the receptor staining step. Add 10 µL of 1,000-fold diluted Live/Dead Fixable Far Red Dead Cell Stain to the cells suspended in 90 µL of PBS, and incubate the vial at 4°C in the dark for 45 min.

c. After the incubation, wash the cells by adding 500 µL of the staining buffer to the vial and centrifuging the vial at 750 × g for 5 min. Aspirate the staining buffer and loosen the cell pellet.

d. Resuspend the cells in 90 µL of PBS and proceed to the receptor staining.

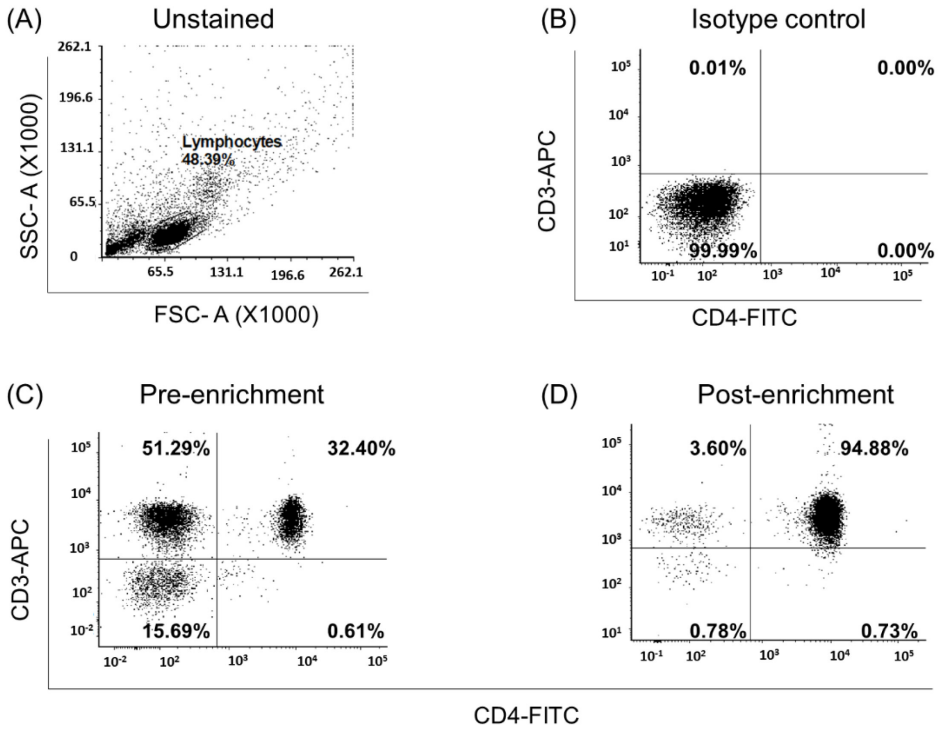


Figure 1. Gating strategy used for analysis of the CD4$^+$ T-cells.
(A) The scatter analysis to identify the lymphocyte population (the gated population). **(B–D)** The lymphocytes population was then gated for CD3$^+$ and CD4$^+$ T-cells. The double-positive population in the upper right quadrant represents the CD4$^+$ T-cells**. (B)** The isotype-control antibodies stain only a tiny proportion of cells, thereby confirming the validity of the staining. Cells falling within the gated zone will be considered CD4$^+$. PBMC **(C)** before and **(D)** after enrichment. While only 32.40% of cells in the pre-enriched PBMC were CD4$^+$, this proportion increased to 94.88% following enrichment. Cells of other lineages reduced concomitantly after the enrichment of the CD4$^+$ cells (compare other quadrants between C and D panels).

D. Activation of the enriched CD4$^+$ T-cells

20. After 3–5 h of incubation, take the 24-well plate from step 10 above out of the incubator and proceed to cell activation.

> *Note: At this stage, the 'activation and no activation' wells each may contain 1 × 10^6–1.5 ×10^6 enriched CD4 cells in 950 µL of complete RPMI medium.*

21. To the 'Activation' well, add 25 µL of PMA at 4 µg/mL (100 ng/mL final concentration) and 25 µL of Ionomycin at 40 µg/mL (1 µg/mL final concentration) solutions. Add 50 µL of plain RPMI medium to the 'No activation' control.
22. Incubate the plate at 37 °C for 12–16 h.

> *Note: Activation will lead to cell clumping compared to the 'no-activation' control.*

E. U-TILDA

The assay involves the distribution of the enriched CD4$^+$ T-cells to several replica wells for each cell

concentration in a 96-well cluster plate. The wells contain the reagents necessary for reverse transcription and PCR amplification. Total RNA will be released into the solution from the cells as the wells are heated, and reverse transcription will be initiated directly without nucleic acid purification. A pool of three RT primers specific to the viral transcript is used in the assay, to preferentially reverse transcribe the viral transcript. Cell-associated impurities do not interfere with the amplification efficiency at the number of cells used in the assay. Following the cDNA synthesis, the virus-specific outer primer pair (N2830 and N2831) will amplify the viral target in the same vial, using the one-step RT-PCR format. The first-round PCR product is diluted and used as the template for the second-round of the nested-PCR amplification, using an inner primer pair (N2832 and N2833), and a fluorescent probe (N2842). The fluorescence intensity of the amplification is monitored in real-time PCR. For additional information regarding the primer design see Mehta *et al*. (2021).

One-step qRT-PCR (First-round of nested-PCR)

23. Mix all the components for the qRT-PCR as shown in Table 2. Prepare the mix for 100 reactions.

> *Note: Master-mix is prepared for 100 samples, including four additional samples to account for sample loss and pipetting errors.*

Table 2. PCR master-mix for the one-step qRT-PCR

Components	Stock Concentration	Final Concentration	Volume (µL)	
			N =1	N =100
Distilled water	-	-	2.8	280.0
Reaction buffer	2×	1×	5.0	500.0
RT primer mix	2 µM	0.1 µM	0.5	50.0
N2830 (FP)	25 µM	0.5 µM	0.2	20.0
N2831 (RP)	25 µM	0.5 µM	0.2	20.0
RNase inhibitor	20 U/µL	0.2 U/µL	0.1	10.0
Superscript III platinum Taq	0.5 U/µL	0.01 U/µL	0.2	20.0
Total volume	-	-	9.0	900.0

24. Dispense 9 µL of the master mix to each well of the 96-well plate, as per the plate layout shown (**Figure 2**).

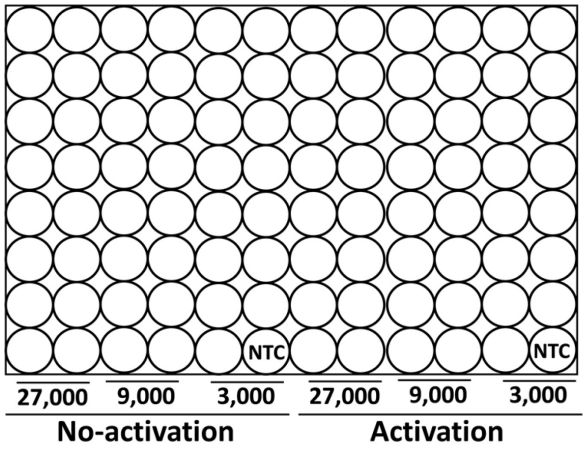


Figure 2. Plate layout for U-TILDA.
The 96-well plate is divided into two halves, 'No-activation' and 'Activation'. Both halves comprise three dilutions of cells as shown, with two columns for each dilution, and one 'No-template' control (NTC).

Cell dilution following activation

25. Following 12–16 h of cell activation, transfer the activated and control cells to two fresh 1.5 mL microcentrifuge tubes appropriately labelled.
26. Determine the cell count.
27. Spin the cells and resuspend the pellet in such a way as to get a cell density of 1 × 10^6 cells in 37 µL of PBS (**Figure 3**). This cell density corresponds to 27,000 cells/µL.
28. Transfer 10 µL of the cell suspension to the next vial containing 20 µL of PBS. This cell density corresponds to 9,000 cells/µL.
29. Repeat the cell dilution, to get a stock of 3,000 cells/µL.

Notes:

a. Sixteen replica wells are used for each cell dilution, with or without cell activation.

b. The minimum number of cells required to set up the assay in the format suggested is 1.62 × 10^6 enriched $CD4^+$ T-cells. These many cells suspended in 37 µL (or 0.81 × 10^6 cells suspended in 30 µL) will correspond to a cell density of 27,000 cells/µL. Cell dilution may be accomplished as suggested in step 28.

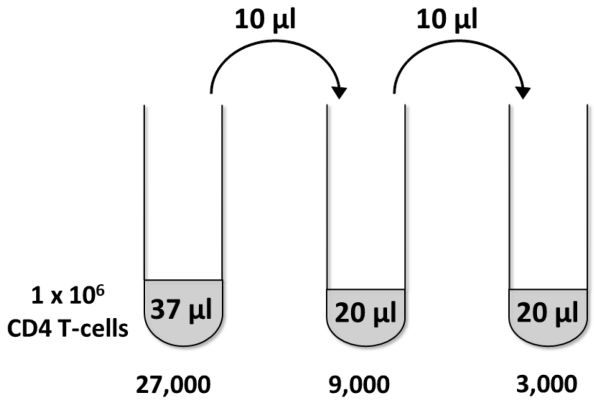


Figure 3. Cell dilution following activation.
One million cells are suspended in 37 µL of PBS (or 0.81 × 10^6 cells in 30 µL) to get a cell density of 27,000 cells per µL. Perform a serial 3-fold dilution as suggested, to get cell stocks of 9,000 and 3,000 cells per µL. The 'no-activation' and 'activation' samples both are diluted using the suggested dilution format.

30. Add 1 µL of the cell suspension from the prepared cell stocks to appropriate wells as per the experimental format (**Figure 2**). Add 1 µL of PBS to the NTC wells.
31. Seal the plate with a sealant film and transfer the plate to a PCR machine.
32. Conduct amplification using the following conditions, reverse transcription at 50°C for 15 min, denaturation at 95°C for 2 min, and 25 cycles of amplification (95°C for 15 s, and 60°C for 4 min).
33. After the amplification, remove the plate from the thermal cycler and dispense 40 µL of TE buffer into each well, to dilute the contents for the second-round of amplification.

Note: The plate with the diluted samples could be stored in a deep freezer for months, if necessary.

Second-round PCR amplification (Real-time format)

34. Prepare the reaction master-mix for the second-round of PCR as shown. The master-mix is sufficient for 100 (96 + 4) reactions (Table 3).
35. Dispense 9 µL of the master-mix to each well of a 96-well PCR plate.

Table 3. PCR mix for the second-round Qpcr

Components	Stock Concentration	Final Concentration	Volume (µL)	
			N = 1	N = 100
Distilled water	-	-	2.50	250.00
Reaction buffer	5×	1×	2.00	200.00
N2832 (FP)	10 µM	2 µM	2.00	200.00
N2833 (RP)	10 µM	2 µM	2.00	200.00
N2842 (probe)	5 µM	0.12 µM	0.25	25.00
MyTaq HS polymerase	5 U/µL	0.12 U/µL	0.25	25.00
Total volume	-	-	9.00	900.00

36. Remove the plate of the first-round of PCR from storage and allow the contents to reach room temperature.
37. Transfer 1 µL of the diluted template from the wells of the first plate to corresponding wells on the second plate.
38. Transfer the second plate to a real-time thermal cycler. Perform PCR using the following program: Pre-incubation at 95°C for 10 min, then 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s.
39. Monitor fluorescence intensity as the amplification progresses.

Notes:

a. The use of a fresh cell lysate without freezing, is expected to make U-TILDA perform with high reproducibility. Châtel et al. suggested the use of the cell lysate directly without storing the samples, to prevent TILDA loss of sensitivity (Châtel et al., 2018).

b. Despite the optimization of the TILDA reported here, additional options are available for further improvisation of the assay. A simple strategy of RNA purification may permit the addition of more RNA to the amplification, thus enabling the interrogation of a larger number of PBMC per reaction well (Pezzi et al., 2017). Unfortunately, an RNA purification step may lead to additional costs, sample loss, and the risk of contamination. Several other DNA polymerases may demonstrate higher levels of tolerance to the presence of impurities in the sample than Taq polymerase (Abu Al-Soud and Rådström, 1998). The use of such enzymes may permit the addition of more RNA to the reaction. The amplification conditions of U-TILDA, including the primer length, annealing temperature, temperature gradation, and the concentrations of the reaction components, have been optimized to the highest extent possible.

Data analysis

The primary aim of any assay attempting to characterize the HIV-1 reservoir is to quantitate the infectious units per million of resting $CD4^+$ T cells. TILDA detects multiply spliced viral transcripts, thus the transcriptionally active proviruses (Mehta *et al*., 2021). However, the expected frequency of infected $CD4^+$ T-cells harboring transcriptionally active events is extremely small, probably one event in 10,000 or 100,000 cells. The assay further assumes that the frequency of such events is uniformly distributed within the cell population, and that a single biologically active cell is sufficient for a positive response from a culture. Given the extremely rare frequency of transcription-competent viral infection events, the assay requires a large number of $CD4^+$ T-cells per well, and by setting up several replica wells per cell dilution. Additionally, to estimate the frequency of transcription-competent viral infection events, reliance on statistical algorithms is essential.

Limiting dilution analyses are based on the Poisson single-hit model, which posits that the number of biologically active particles in each culture varies according to a Poisson distribution. The best estimate for the limiting dilution analysis can be calculated using the maximum-likelihood estimate (MLE) (Ahmad and Parvaiz, 2020). MLE is a method for estimating the parameters of a probability distribution based on observed data. This is accomplished by maximizing a probability function, such that the observed data is most probable under the assumed statistical model. The MLE is obtained using a computational algorithm, and there are many software programs available based on this algorithm. We use the ELDA software developed by Walter + Eliza Hall Bioinformatics Institute of Medical Research for the analysis (Hu and Smyth, 2009).

1. Export the qPCR data to an excel sheet.
2. Identify the number of replicate wells positive for amplification at each dilution independently, in both 'No activation' and 'Activation' controls.
3. Following the PCR amplification, tabulate the results demonstrating the number of positive wells under each experimental condition (**Figure 4A**).
4. Access the ELDA homepage at https://bioinf.wehi.edu.au/software/elda/ and follow instructions to enter the experimental data.

Notes:

*a. The software will provide the estimated frequency of the positive events (the lower, upper, and mean estimates) of the cell population (**Figure 4C**).*

b. The total number of cells harboring transcription-competent proviruses per million $CD4^+$ T-cells may be calculated by dividing 1×10^6 with the mean estimate obtained using the software.

No-activation **Activation**

(A) Data tabulation

27,000		9,000		3,000	
12.5	16.96	N/A	N/A	36.28	N/A
N/A	19.32	28	38.89	N/A	N/A
14.67	N/A	N/A	N/A	N/A	16.79
N/A	16.26	N/A	21.25	24.16	N/A
14.19	13.02	N/A	N/A	41.27	N/A
N/A	N/A	N/A	N/A	N/A	N/A
15.74	14.78	N/A	N/A	N/A	N/A
N/A	N/A	N/A	N/A	N/A	NTC

27,000		9,000		3,000	
14.91	11.7	14.31	12.57	N/A	17.85
12.13	11.43	13.09	12.43	17.99	17.8
11.58	15.23	13.33	17.72	17.05	18.45
12.23	10.78	14.22	16.46	N/A	16.89
12.28	13.48	16.39	17.18	15.11	16.45
12.19	14.14	18.15	17.11	13.58	14.45
12.44	13.9	14.83	18.31	11.19	17.74
11.83	16.27	17.28	11.85	20.18	NTC

(B) Data summarization

Cells/well	Replicate wells	Positive wells
27,000	16	9
9,000	16	3
3,000	15	4

Cells/well	Replicate wells	Positive wells
27,000	16	16
9,000	16	16
3,000	15	13

(C) Estimated frequency of the positive events

Estimate			No. of positive events /10^6 cells
Upper	Lower	Mean	
17,291	47,900	28,779	35

Estimate			No. of positive events /10^6 cells
Upper	Lower	Mean	
797	2,662	1,457	686

Figure 4. Quantitating the frequency of cells producing viral msRNA.

(A) Tabulated qPCR data. The numbers represent the Ct value of each well after the PCR. N/A, no amplification.

The data of subject S338_02 are reproduced from a previous publication (Mehta *et al*., 2021). **(B)** The fraction of wells positive among the total number of replicate wells is summarized for each cell number. Note that an additional round of cell dilution may be warranted as 13 of 15 wells under 'activation' are positive in PCR. **(C)** The software returns the estimated frequency of the positive events of the cell population. The number of transcription-competent viral infection events in the enriched $CD4^+$ T-cells was estimated to be 35 and 686 before and after cell activation, respectively. Thus, approximately a 20-fold enhancement in the number of infected cells producing viral transcripts was observed under the activation conditions described above.

Recipes

1. **Revival media**
   RPMI medium, 2% FBS, 20 mM HEPES, and 1 mM EDTA

2. **Enrichment media**
   DPBS, 2% FBS, and 1 mM EDTA

3. **Staining buffer**
   2% FBS in PBS

4. **RPMI medium supplemented with10% FBS**
   RPMI medium, 10% FBS, 100 units/mL penicillin G, 2 mM L-glutamine, and 100 µg/mL streptomycin

5. **Phosphate-buffered saline (PBS) solution**
   137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4

6. **Tris-EDTA (TE) buffer**
   10 mM Tris-HCl and 1 mM EDTA•Na_2

7. 40 µg/mL PMA
   0.4% of 1 mg/mL PMA stock in RPMI medium

8. **4 µg/mL Ionomycin**
   4% of 1 mg/mL Ionomycin stock in RPMI medium

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Competing interests

The authors declare that there is no conflict of interest or competing interest regarding the publication of this article.

Ethics

The Human Ethics and Biosafety Committee of Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR), Bangalore, reviewed the proposal and approved the study. Written informed consent was obtained from all study participants following the approval of the institutional review board.

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