bio-protocol

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CD8 T Cell Virus Inhibition Assay Protocol

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Abstract

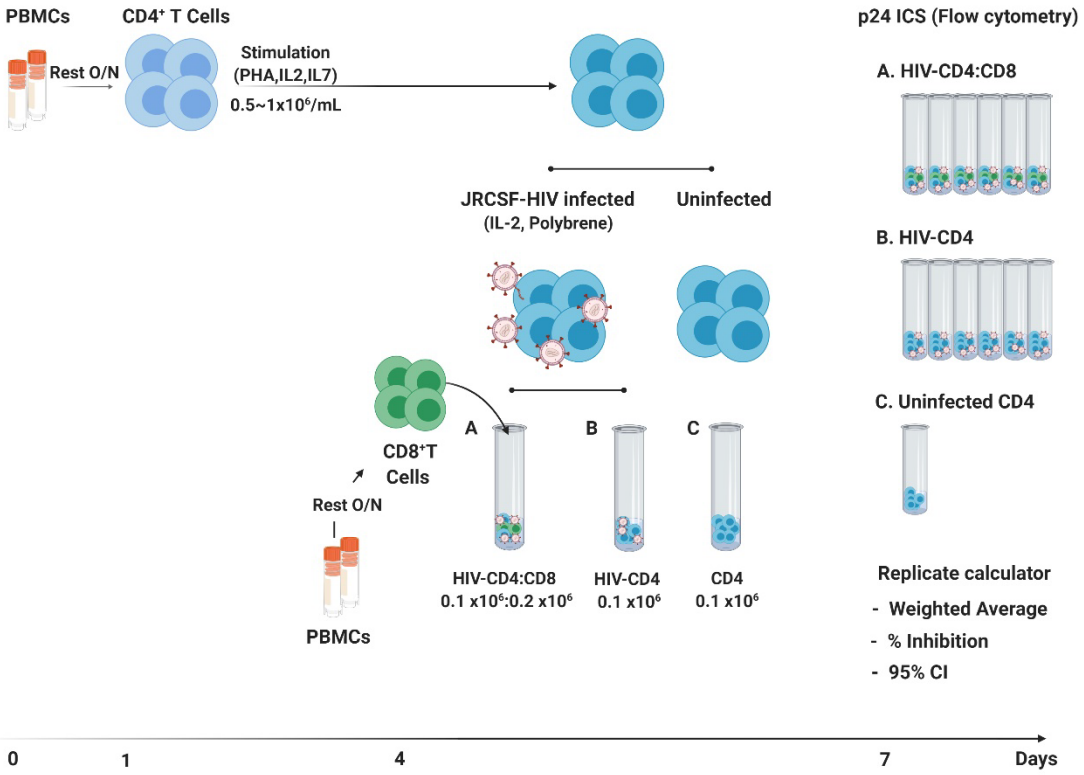
The human immunodeficiency virus (HIV)-1 viral inhibition assay (VIA) measures $CD8^+$ T cell-mediated inhibition of HIV replication in $CD4^+$ T cells and is increasingly used for clinical testing of HIV vaccines and immunotherapies. Different VIAs that differ in length of CD8:CD4 T cell culture periods (6–13 days), purity of CD4 cultures [isolated $CD4^+$ T cells or $CD8^+$ depleted peripheral blood mononuclear cells (PBMCs)], HIV strains (laboratory strains, isolates, reporter viruses) and read-outs of virus inhibition (p24 ELISA, intracellular measurement of p24, luciferase reporter expression, and viral *gag* RNA) have been reported.

Here, we describe multiple modifications to a 7-day VIA protocol, the most impactful being the introduction of independent replicate cultures for both HIV infected-CD4 (HIV-CD4) and HIV-CD4:CD8 T cell cultures. Virus inhibition was quantified using a ratio of weighted averages of $p24^+$ cells in replicate cultures and the corresponding 95% confidence intervals. We identify methodological and analysis changes that could be incorporated into other protocols to improve assay reproducibility. We found that in people living with HIV (PLWH) on antiretroviral therapy (ART), CD8 T cell virus inhibition was largely stable over time, supporting the use of this assay and/or analysis methods to examine therapeutic interventions.

Keywords: CD4, CD8, HIV, JRCSF, T-cell, p24, Virus inhibition

This protocol was validated in: Front Immunol (2021), DOI: 10.3389/fimmu.2021.666991

Graphic abstract:



Background

A CD8$^+$ T cell virus inhibition assay (VIA) measures the *in vitro* ability of CD8$^+$ T cells to inhibit human immunodeficiency virus (HIV)-1 replication in autologous CD4$^+$ T cells. This assay captures the full range of CD8$^+$ T cell anti-viral activity in response to *in vitro* antigen presentation of virus-derived peptides by infected CD4 T cells. VIAs are increasingly used in clinical studies but mostly as an exploratory assay, because the complex co-culture of the VIA has substantial assay variability and a limited dynamic range. Different VIAs have been reported (Yang, O. O. *et al.*, 1997; Sáez-Cirión *et al.*, 2009, 2010; Spentzou *et al.*, 2010; Julg *et al.*, 2010; Freel *et al.*, 2011; Yang, H. *et al.*, 2012, 2013; Naarding *et al.*, 2014; Slichter *et al.*, 2014; Hancock *et al.*, 2015) that differ in length of CD8:CD4 T cell culture periods (6–13 days), purity of CD4 cultures (isolated CD4$^+$ T cells or CD8$^+$ depleted PBMCs), HIV strains (laboratory strains, isolates, reporter viruses), and read-outs of virus inhibition (p24 ELISA, intracellular measurement of p24, luciferase reporter expression, and viral *gag* RNA). Here, we describe multiple modifications to a 7-day VIA protocol, the most impactful being the introduction of independent replicate cultures for both HIV infected-CD4 (HIV-CD4) and HIV-CD4:CD8 T cell cultures. Virus inhibition was quantified using a ratio of weighted averages of p24$^+$ cells in replicate cultures and the corresponding 95% confidence intervals (CI). We found that in people living with HIV (PLWH) on antiretroviral therapy (ART), CD8$^+$ T cell virus inhibition was largely stable over time, supporting the use of this assay and/or analysis methods to examine therapeutic interventions.

Materials and Reagents

1. Falcon™ Round-Bottom Polystyrene Test Tubes (Falcon™, catalog number: 352054)
2. Falcon 50 mL Conical Centrifuge Tubes (Corning, catalog number: 352070)
3. Falcon 15 mL Conical Centrifuge Tubes (Falcon™, catalog number: 352097)
4. Phytohaemagglutinin (PHA) (Sigma-Aldrich, catalog number: L8902-5MG)
5. Benzonase® (Sigma-Aldrich, catalog number: Nuclease E1014)
6. $CD4^+$ T Cell Isolation Kit (MACS, Miltenyi-Biotec, catalog number: 130-096-533)
7. HIV-1 JR-CSF Infectious Molecular Clone (pYK-JRCSF) NIH reagent program
8. IL-2 (Prometheus Proleukin, Aldesleukin, catalog number: 407682M)
9. IL-7 (PeproTech, catalog number: 200-07-10UG)
10. Polybrene (Santa Cruz Biotechnology, catalog number: NC9840454)
11. RPMI (Corning®, catalog number: 10-040-CV)
12. FBS (VWR Life Science, catalog number: 97068-091)
13. L-Glutamine (Corning™, catalog number: 25005CI)
14. Sodium Pyruvate (Corning™, catalog number: 25-000-CIR)
15. Penicillin-streptomycin (Gibco™, catalog number: 15070063)
16. HEPES (Corning™, catalog number: 25-060-CI)
17. PBS (Corning™, catalog number: 21-030-CM)
18. BSA (Sigma, catalog number: A8412)
19. EDTA (Corning™, catalog number: 46-034-CI)
20. Lysolecithin (Sigma, catalog number: L4129-25MG)
21. Paraformaldehyde (PFA) (Santa Cruz Biotechnology, catalog number: sc-281692)
22. Methanol (Sigma, catalog number: 34860-1L-R)
23. Nonidet P-40 (Biotang Inc, catalog number: BTBB914)
24. Zombie NIR Fixable Viability Kit (Biolegend, catalog number: 423106)
25. p24-FITCKC57-FITC (catalog number: 6604665, Beckman)
26. CD3-BV421 (BD Biosciences, catalog number: BDB562426)
27. CD4-AF488 (Biolegend, catalog number: 317434)
28. CD8-BV510 (Biolegend, catalog number: 344732)
29. Anti-mouse Ig, κ compensation beads (BD™, catalog number: 552843)
30. PBMCs: Isolated from PLWH receiving antiretroviral therapy (HIVART) and seronegative individuals (healthy donor (HD))
31. R-10+ (see Recipes)
32. R-20+ (see Recipes)
33. Cell isolation buffer (see Recipes)
34. 50% Methanol/PBS (see Recipes)
35. 0.1% NP-40/PBS (see Recipes)

Equipment

1. Centrifuges (Thermo Scientific, ST40R TX-1000, catalog number: 50144036)
2. Muse Cell Analyzer (EMD Millipore Corporation, catalog number: 0500-3115)
3. Incubator (Thermo Scientific, catalog number: 190408340396)
4. MACS magnet (Miltenyi Biotech, OctoMACS Separator, catalog number: 130-042-108)
5. Flow Cytometer (BD LSRFortessa)
6. FINNPIPETTE F1 GLP (Thermo Scientific, catalog number: 4700850N)

Software

1. Excel (Microsoft)
2. Flow Jo (Flow Jo, LLC/ FlowJo_v10.8.0, https://www.flowjo.com/)

Procedure

Note: All procedures were performed in a BSL2+ space under a BSC sterile environment.

Day 0: Thaw PBMCs for CD4$^+$ T cell targets

1. Make up 25 units/mL Benzonase in R-10+ and warm this solution in a 37°C incubator. Prepare ~10 mL per vial (1 mL) of frozen PBMCs, to wash off the DMSO.
2. Rapidly thaw PBMCs in a 37°C water bath, then transfer cell contents into prewarmed R10+ with benzonase (25 unit/mL) (1 vial into 10 mL), wash three times with R-10+ by spinning at for at 500 × *g* and room temperature (RT) for 5 min, and count. Resuspend at 2 × 10^6 cells/mL in R-20+ and place in a 37°C humidified CO_2 incubator overnight. See **Figure 1** for the procedure on day 0, and see **Table 1** for PBMC requirements.

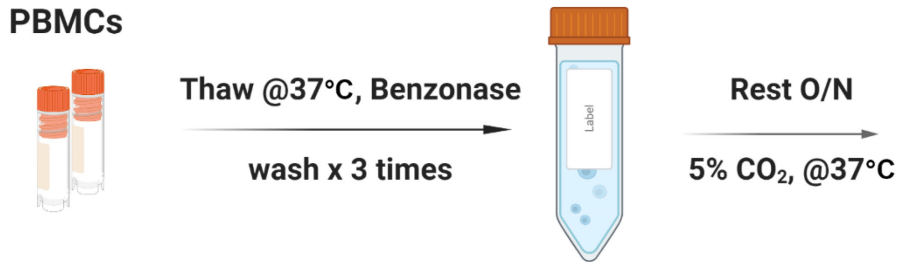


Figure 1. Procedure for Day 0

Table 1. PBMC estimates for VIA[a]

VIA PBMC requirements [b]	1 timepoint	4 timepoints[c]
Targets	10^7	3–4 ×10^7
Effectors (2:1)	10^7	10^7/timepoint

[a]Estimates based on JRCSF infection ranging 1–10% and PBMC viability >85%, Using 0.1 × 10^6 CD4$^+$ T cells per replicate, total 4–6 replicates.
[b]Assume 6 CD4-HIV and 6 CD4-HIV:CD8 co-cultures and 1 uninfected control.
[c]One set of CD4-HIV-only and 1 uninfected CD4 control will be used for calculations of percentage of inhibition across multiple timepoints.

Notes:

1. *Benzonase decreases cell clumping, improving cell recovery.*
2. *Negative Isolation of CD4+ T cells produced lower non-specific virus inhibition in HIV-negative donors than assays performed with CD8-depleted PBMC.*

Day 1: Isolate CD4$^+$ T cell targets

1. Negatively isolate CD4$^+$ T cells from thawed PBMCs using MACS beads as per manufacturer's instructions. A typical isolation provides >96% CD4$^+$ T cell purity.
2. Count and suspend cells at 2 × 10^6/mL in R-10+ media containing IL-2 (20 IU/mL), IL-7 (5 ng/mL), and either 5 µg/mL PHA (PLWH ± ART) or 3 µg/mL (HIV seronegative donor). See **Figure 2** for the procedure on day 1.
3. Plate the cells as listed, and culture for 72 ± 3 h in a 37°C incubator with 5% CO_2:
   - 5 × 10^6 cells/well in 6-well plate (in 2.5–3.0 mL/well)
   - 3 × 10^6 cells/well in 12-well plate (in 1.5–2.0 mL/well)
   - <1.5 × 10^6 cells/well in 24- or 48-well plates (in 1.0–1.5 mL/well)

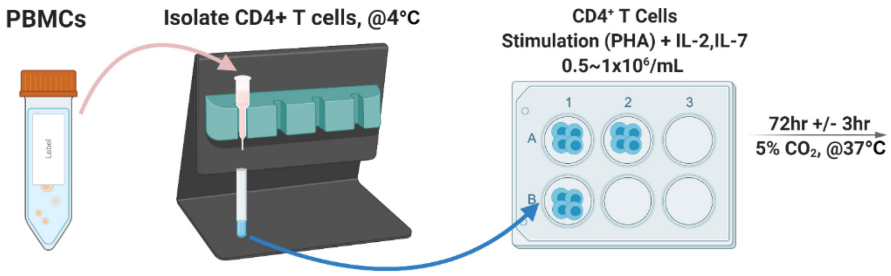


Figure 2. Procedure for Day 1

Notes:

1. *VIA assays using CD8-depleted PBMC result in higher infectivity (%p24$^+$ cells) in our hands. However, CD4$^+$ T cells are recommended over CD8-depleted PBMCs because of improved assay reproducibility, possibly due to the exclusion of CD56$^+$ NK cells, and more consistent CD4:CD8 T cell ratios.*
2. *IL-2+IL-7 results in higher %p24$^+$ infection, possibly due to IL-7-mediated increase of CCR5 expression on CD4$^+$ T cells (Llano et al., 2001).*
3. *Final CD4$^+$ T cell concentrations can be 1–2 × 10^6 depending on volume capacity in each well. Generally, the cell recovery after 72 h (Day 4) stimulation ranges between 70–120% of the input cells, and viability ranges between 70–90%.*
4. *Different PHA concentrations are used because PHA >3 µg/mL in seronegative donors results in significant cell loss. Cell loss following >3 µg/mL PHA stimulation is also observed in HIV+ donors, but the 5 µg /mL stimulation improves HIV %infection.*

Day 3: Thaw PBMCs for CD8$^+$ T cell effectors

1. Make 25 unit/mL Benzonase in R-10+ and warm in a 37°C incubator.
2. Rapidly thaw PBMCs in a 37°C water bath, then transfer cell contents to prewarmed R-10+ with benzonase (25 unit/mL), wash three times with R-10+ by spinning at 500 × *g* and RT for 5 min, and count. Resuspend in 2 × 10^6/mL in R-20+ and place in a 37°C, humidified CO_2 incubator overnight. See **Table 1** for PBMC needs.

Day 4: HIV infection of target CD4$^+$ T cells

1. After 72 ± 3 h stimulation with PHA, harvest mitogen activated CD4$^+$ targets from wells using a sterile disposable pipette, and wash three times with >10 mL of R-10+ to remove PHA in culture.
2. Count cells and resuspend to 10^7 cells/mL in a 15 mL Falcon tube in R-10+ supplemented with IL-2 at 20 IU/mL.

   Note: Generally, the cell recovery after 72 h (Day 4) stimulation ranged between 70–120% of the input cells, and viability ranged between 70–90%.

3. Transfer 10^5 cells to a flow cytometry tube for use as uninfected control.

4. Infect the remaining $CD4^+$ T cells with $HIV\text{-}1_{JRCSF}$ (final MOI of 0.03).
   a. Supplement R-10+ with IL-2 at 20 IU/mL and Polybrene at 8 μg /mL.
   b. Dilute the virus stock in Polybrene/IL-2/R-10+ media to a 2× final concentration.
   c. Add an equal volume of 2× virus to $CD4^+$ targets, resulting in a final cell concentration of 5×10^6/mL. Do not exceed more than 2×10^6 cells per 15 mL Falcon tube (0.4 mL).
   d. Spin-oculate at 1,200 × *g* and 27°C for 2 h with brake.
   e. Following spin-oculation, wash three times with R-10+ to remove residual virus and polybrene.
5. Count and resuspend cells at 1×10^6/ mL, by adding R-10+ supplemented with IL-2 at 20 IU/mL.

Notes:
1. *Counting accuracy is critical in this step, for ensuring the correct ratio of $CD4^+$ targets to $CD8^+$ effectors. Counting in triplicates is recommended with a coefficient of variance (CV) <10%.*
2. *Addition of polybrene increases JRCSF infection by up to three fold, but does not improve NL4.3 infection.*

Day 4: Prepare $CD8^+$ T cells effectors

1. While $CD4^+$ T cells are spin-oculating, isolate $CD8^+$ T cells from rested overnight PBMCs using $CD8^+$ MACs microbeads (positive selection), following the manufacturer's instructions.
2. Count and resuspend isolated $CD8^+$ T cells at 2×10^6/mL in R-10+ with IL-2 at 20 IU/mL.

Note: Again, counting accuracy is critical in this step for ensuring correct ratio of $CD4^+$ targets to $CD8^+$ effectors. Counting in triplicates and a CV <10% is recommended.

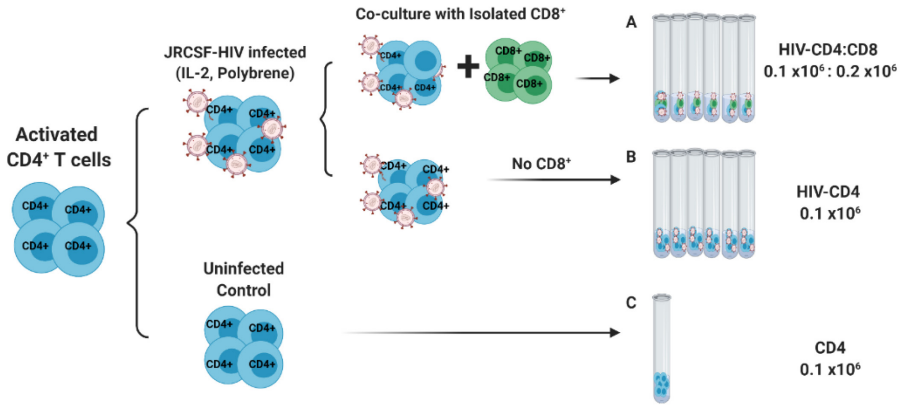


Figure 3. Procedure for Day 4

Day 4: Effector: Target co-cultures

1. Cell numbers permitting, set up the following labeled flow cytometry tubes:
   - 6 tubes containing 10^5 $CD4^+$ targets (100 μL) + 100 μL R-10+
   - 6 tube containing 10^5 $CD4^+$ targets (100 μL) + 2×10^5 (100 μL) $CD8^+$ T cell effectors
   - 1 tube containing 10^5 uninfected $CD4^+$ cells (100 μL) + 100 μL R-10+
2. Place co-cultures in a 37°C, humidified CO_2 incubator 3 days. See **Figure 3** for the procedure on day 4.

Notes:

1. *To minimize the variation, $CD8^+$ and $CD4^+$ T cells were first combined 2:1, and then divided across flow cytometry tubes in a total volume of 200 µL/tube, containing 3 × 10^5 cells.*
2. *This is day 0 of HIV-1 JRCSF super-infection and effector target co-culture. Peak of infection (p24%) is at day 3.*
3. *If using different E:T ratios, adjust accordingly. We recommend ensuring the final culture volume across all tubes is kept consistent.*
4. *Sterile flow cytometry tubes are recommended over 48- and 96-well plates for cultures containing 1–3 × 10^5 cells because of better infectivity, less cell loss, and time savings.*

Day 7: Intracellular HIV-specific p24 staining protocol

Notes:

1. *Peak $\%p24^+$ cells are observed 3 days post-infection (data not shown).*
2. *Cell cultures are directly stained in the same flow cytometry tubes. We recommend using printed labels for flow tubes (not handwritten).*

Table 2. Samples/controls for p24 staining

Tube	Detail	Replicates
Live Dead control	Heat-kill cells in a 70°C water bath, for 10 min	1
Controls	CD4 FMO recommended as infection decreases CD4 expression	1
	Non-stained control	1
Test Samples	Uninfected control CD4-only[a]	1
	Infected $CD4^+$ targets	6[b]
	Co-culture: Infected $CD4^+$ targets + Effector $CD8^+$	6[b]

[a] Used to define $p24^+$ gate; in PLWH, this control is also critical to examine endogenous viral replication. In untreated HIV infection, replicates should be considered.
[b] See main manuscript text (Xu *et al*., 2021; DOI: 10.3389/fimmu.2021.666991) for further details on calculation of replicate number.

1. Spin cells down at 500 × *g* and RT for 5 min.
2. Wash cells with 2 mL of PBS, spin cells down at 500 × *g* and RT for 5 min, and resuspend cells in 100 µL of PBS.
3. Add 0.2 µL of Zombie NIR™. Wrap the tube with foil and incubate for 20–30 min at RT.
4. During this incubation
   a. Bring lysolecithin fixative to RT.
   b. Prepare an ice bucket.
   c. Bring centrifuge temperature to 4°C.
5. Add 2 mL of PBS per tube and centrifuge at 500 × *g* and RT for 5 min. Decant excess supernatant onto a paper towel.
6. Fix cells by adding 1 mL of 20 µg/mL lysolecithin in 4% paraformaldehyde.
7. Vortex. Incubate for 2 min at RT.
8. Centrifuge at 500 × *g* and 4°C for 5 min. Decant or aspirate supernatant. Vortex.
9. Add 1 mL of cold 50% methanol (-10 to -20°C).
10. Vortex. Incubate on ice for 15 min.
11. Centrifuge at 500 × *g* and 4°C for 5 min. Decant or aspirate supernatant. Vortex.
12. Add 1 mL of 0.1% NP-40 (2–8°C).
13. Vortex vigorously. Incubate on ice for 5 min.

14. Centrifuge at 500 × *g* and 4°C for 5 min. Decant or aspirate supernatant. Vortex.
15. Make up an antibody mastermix. Per tube, add 2 µL each of p24 (5 µg/mL), CD3 (2 µg/mL), CD4 (2 µg/mL), and CD8 (2 µg/mL) antibodies.
16. Add antibody mastermix to tubes and vortex. Incubate at RT for 15 min .
17. Add 2 mL of PBS. Centrifuge at 500 × *g* and RT for 5 min. Decant or aspirate supernatant. Vortex.
18. Prepare compensation controls for each fluorochrome.
19. Acquire on a flow cytometer within 6 h. See **Figure 4** For the gating scheme.

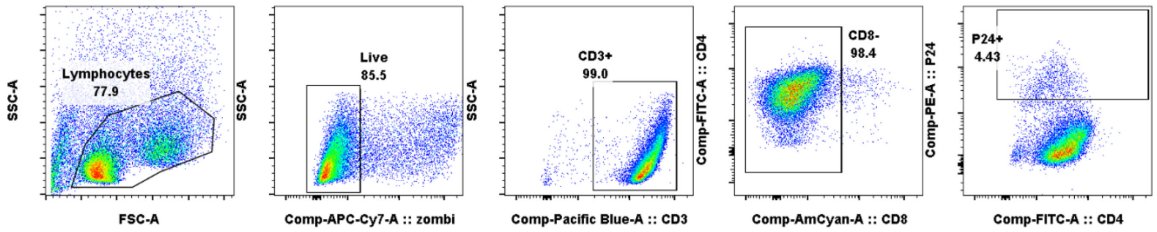


Figure 4. Gating Scheme.
The frequency of infected $CD4^+$ T cells was defined as the percentage of HIV-1 $p24^+$ cells among live single $CD3^+$ $CD8^-$ lymphocytes, using the uninfected $CD4^+$ cell culture that had a $p24^+$ frequency <0.1 across all assays.

Notes:
1. *An acquisition rate <1,000 events/sec is recommended, given the low frequency of events.*
2. *Set $CD4^+$ >20,000 events or %p24 >500 events in $CD4^+$ target alone as criteria (Xu et al., 2021).*

Data analysis

Practical changes to the VIA, such as cell culture in flow cytometry tubes (detailed protocol above), enabled increased assay throughput, which is likely to be beneficial for clinical testing. In our hands, the introduction and acquisition of independent culture replicates greatly improved assay confidence. An Excel template is provided with this manuscript, to facilitate easy calculation of percentage of virus inhibition and a corresponding 95% CI (VIA Replicate Calculator, https://github.com/glab-hiv/via). These calculations have a broader application in flow cytometry, where replicate assays acquire different cell numbers. We recommend that investigators consider calculation of percentage inhibition via weighted averages and 95% CI, using the template provided. Weighted averages and 95% CI could also be used for analysis of independent replicates in standard functional intracellular cytokine staining (ICS).

Simple tables (Tables 3 and 4) are provided to help determine the level of HIV infection needed and the minimum number of $p24^+$ cells that must be acquired to achieve 90% statistical power in the detection of $CD8^+$ T cell mediated inhibition of HIV replication. Again, the values produced in simulations for these tables have broader application in flow cytometry, where low frequency cells are being measured to define the minimum cell numbers and/or replicates required to have statistical power to detect a difference in functional response (*e.g.*, frequency of cytokine producing cells).

Table 3. Minimum required percentage of $p24^+$ cells for 90% statistical power.
At 90% statistical power, the minimum percentage of $p24^+$ cells (in HIV-CD4 T cell cultures) needed to observe a threshold virus inhibition of 5–30% for 2–6 replicates. The threshold of 8% inhibition is the receiver operating characteristic (ROC)-determined cut-off of specific $CD8^+$ T cell-mediated virus inhibition from study data. Percentages of $p24^+$ cells were generated using simulations (500 iterations) based on parameters from the real data. The minimum allowed percentage of HIV infection was 0.5%.

	VIRUS INHIBITION (%)						
NUMBER OF REPLICATES	5	8	10	15	20	25	30
2	14.99	7.49	4.78	1.98	1.05	0.61	0.5
3	11.84	4.63	3.16	1.3	0.67	0.5	0.5
4	8.91	3.69	2.3	0.95	0.5	0.5	0.5
5	7.79	3.17	1.88	0.79	0.5	0.5	0.5
6	6.97	2.43	1.57	0.66	0.5	0.5	0.5

Table 4. Minimum required number of $p24^+$ cells for 90% statistical power.
At 90% power, the minimum number of $p24^+$ cells (denominator of 20,000 $CD4^+$ T cells) needed to observe a threshold virus inhibition of 5–30% for 2–6 replicates. The minimum allowed percentage of HIV infection was 0.5%.

	VIRUS INHIBITION (%)						
NUMBER OF REPLICATES	5	8	10	15	20	25	30
2	2999	1498	957	397	209	122	100
3	2367	926	632	259	135	100	100
4	1783	738	459	191	100	100	100
5	1559	635	375	159	100	100	100
6	1393	486	314	133	100	100	100

Recipes

1. R-10+ (store at 2–8°C, limit light exposure)

RPMI-1640
10% FBS
2 nM L-glutamine
1 mM sodium pyruvate
1× penicillin-streptomycin
10 mM HEPES

2. R-20+ (store at 2–8°C, limit light exposure)

RPMI-1640
20% FBS
2 nM L-glutamine
1 mM sodium pyruvate
1× penicillin-streptomycin
10 mM HEPES

3. Cell isolation buffer (store at 2–8°C)

PBS
0.5% BSA
2 mM EDTA

4. 20 µg/mL lysolecithin in 4% PFA [#] (store at 2–8°C)

Dissolve 10 mg lysolecithin in 500 mL of the 4% PFA solution.

5. 50% Methanol/PBS[#] (store at -20°C)

6. 0.1% NP-40/PBS* (store at 2–8°C)

Add 0.5 mL of NP-40 to 499.5 mL of PBS.

*Store in single use aliquots at -80°C.
[#]Note: The above reagent solutions have a minimum shelf life of 6 months, if stored as indicated.

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Competing interests

The authors declare no conflict of interest.

Ethics

Peripheral blood mononuclear cells (PBMCs) were isolated from PLWH receiving antiretroviral therapy (HIVART) and seronegative individuals (healthy donor (HD)). All HIVART participants were receiving stable standard-of-care ART and had maintained plasma HIV-1 RNA < 50 copies/mL and a CD4 T cell count of > 300/μL for at least 6 months before enrollment. All experimental protocols were approved by local Institutional Biomedical Review Boards (ethics numbers: 14-0741, 11-0228, 13-3613, 12-1660, and 10-01330) and performed in accordance with the relevant guidelines. HD used for assay standardization were recruited by the UNC CFAR HIV/STD Laboratory Core (IRB 96-0859, http://unccfar.org/portfolio/hiv-std-laboratory-core/) and New York Blood Center (https://nybloodcenter.org).

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