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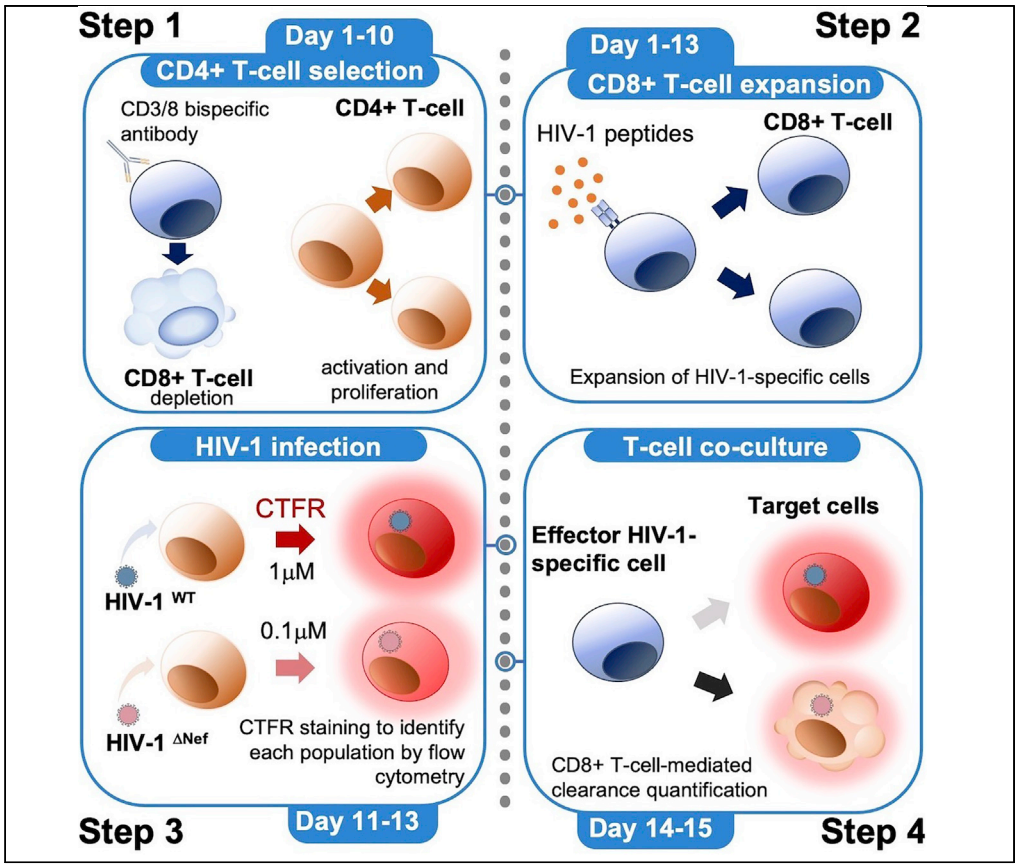
Viral competition assay to assess the role of HIV-1 proteins in immune evasion



CD8+ T lymphocytes can recognize and eliminate cells infected by viruses. However, the human immunodeficiency virus (HIV-1) has developed mechanisms to evade CD8+ T-cell-mediated clearance. Here, we describe a protocol to assess the role of the HIV-1 protein Nef in immune evasion. The viral competition assay reveals the preferential killing of HIV-1-infected cells unable to express Nef. This methodology can be extended to study HIV-1 proteins involved in immune evasion and viral variants encoding cytotoxic T lymphocyte escape mutations.

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

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Highlights

The viral competition assay reveals the role of the viral protein Nef in immune escape

From genomic DNA to sequencing-ready libraries in approximately one day

Less than 1×10^7 PBMCs are required to perform the viral competition assay

Duette et al., STAR Protocols 4, 102025
March 17, 2023
https://doi.org/10.1016/j.xpro.2022.102025

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Viral competition assay to assess the role of HIV-1 proteins in immune evasion

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https://doi.org/10.1016/j.xpro.2022.102025

SUMMARY

CD8+ T lymphocytes can recognize and eliminate cells infected by viruses. However, the human immunodeficiency virus (HIV-1) has developed mechanisms to evade CD8+ T-cell-mediated clearance. Here, we describe a protocol to assess the role of the HIV-1 protein Nef in immune evasion. The viral competition assay reveals the preferential killing of HIV-1-infected cells unable to express Nef. This methodology can be extended to study HIV-1 proteins involved in immune evasion and viral variants encoding cytotoxic T lymphocyte escape mutations.

For complete details on the use and execution of this protocol, please refer to Duette et al. (2022).[1]

BEFORE YOU BEGIN

1. The protocol below describes the specific steps for using the two viral strains: HIV-NL4-3 (HIV^{WT}) and Nef-deleted HIV-NL4-3 ($HIV^{\Delta Nef}$, see key resources table). However, this method can also be performed using:
   a. HIV-1 constructs with deletions for different viral proteins involved in immune evasion or,
   b. viral variants containing cytotoxic T lymphocyte (CTL) escape mutations in Major Histocompatibility Complex class I (MHC-I) restricted epitopes.
2. The samples used to develop this protocol were obtained from a specific cohort of individuals living with HIV-1 on long-term antiretroviral treatment.[1] Therefore, samples obtained from different cohorts of HIV-1-infected participants may result in different outcomes in terms of CD8+ T-cell-mediated killing efficiency. However, a similar methodology was previously applied showing similar level of CD8+ T-cell-mediated killing using a different cohort of HIV-1-infected participants.[2]

Institutional permissions

Samples and experiments performed for this manuscript were undertaken in accordance with the Declaration of Helsinki, with Ethics approval from the institutional review boards at the University of New South Wales and the Western Sydney Local Health District.

Peripheral Blood Mononuclear Cells (PBMCs) from HIV-1 infected individuals are necessary to perform the described protocol, therefore, permission from the relevant institution/s is required.

Note: All incubation steps at 37°C indicated in this protocol should take place in a 5% CO_2 cell culture incubator.

Note: Cell counting was performed by staining the indicated cell suspension with trypan blue 0.4%. Cells were loaded into a hemacytometer and examined immediately under a microscope at low magnification.

Note: This protocol involves the manipulation of HIV-1 stocks and samples from individuals infected with HIV-1, therefore all the lab work must be undertaken in Biosafety Level 3 (BSL-3) laboratories.

Plasmid preparation

Timing: 3 days

3. All plasmids necessary for this protocol can be prepared by applying standard protocols for bacterial transformation and plasmid isolation.
   a. We recommend using MAX Efficiency™ Stbl2™ Competent Cells to clone the plasmids.
   b. and NucleoBond Xtra Midi kit for plasmid purification.

Note: All the plasmids used in this protocol were resuspended in TE (10 mM Tris/HCL, 1 mM EDTA) buffer according to the kit recommendation.

Preparation of viral stocks

Timing: 7 days

4. 2 Days before plating cells: Thaw and prepare HEK 293T cells.
   a. Warm 5 × 10^6/mL cryogenically frozen HEK 293T cells in a 37°C water-bath.
   b. Before cells are completely thawed, transfer 1 mL of thawed cells into 15 mL Falcon tube containing 9 mL pre-warmed DMEMc.
   c. Centrifuge cells at 300 × *g* for 5 min and remove the supernatant.
   d. Resuspend the pellet in 10 mL of fresh pre-warmed DMEMc and transfer the cells into a 100 mm cell culture dish.
   e. Incubate cells for 2 days at 37°C.

Note: We recommend using fresh HEK 293T cells for every viral stock production. The efficiency of transfection of these cells may be affected if cells cultured for a long time are used.

5. Day 1: Plate HEK 293T cells.

⚠ CRITICAL: HEK 293T cells tend to detach from the culture dish or plate surface with little agitation. Avoid aggressive pipetting and be gentle when washing cells or replacing the culture media.

   a. Gently remove the supernatant and add 5 mL of D-PBS.
   b. Remove the D-PBS and add 3 mL of TrypLE express (see key resources table) to detach the cells.
   c. Incubate cells for 5 min at 37°C.
   d. Add 7 mL of DMEMc and transfer the detached cells into a 15 mL Falcon tube.
   e. Pellet the cells by centrifugation for 5 min at 300 × *g*.
   f. Pour off the supernatant and resuspend the cell pellet adding 10 mL DMEMc.
   g. Perform cell counting and plate 3 × 10^5 cells in 2 mL of DMEMc per well in a 6-well plate.

Note: At least 2 wells with cells are necessary to prepare the HIV^{WT} and $HIV^{\Delta Nef}$ viral stocks (one well per viral stock). The number of cells/wells needed will depend on the number of experiments to be performed.

h. Incubate cells 12–18 h at 37°C.

Note: Infection of primary CD4+ T-cells with HIV-1 can be challenging. To overcome this challenge, we recommend making VSV-G-pseudotyped HIV-1 viral stocks as described below. Producing viral particles harboring VSV-G envelope significatively increases HIV-1 infectivity by improving viral uptake by the target cells.[3]

6. Day 2: Transfection of HEK 293T cells.
   a. Prepare transfection master mix in two 1.5 mL Eppendorf tubes. Each tube should contain:
      i. 200 µL Opti-Mem.
      ii. 0.4 µg pHEF-VSV-G plasmid.
      iii. 1 µg HIV-1 $NL4\text{-}3^{AD8ENV}$ (HIV^{WT}) plasmid or $HIV^{NEF\text{-}ve}$ ($HIV^{\Delta Nef}$) plasmid.
      iv. 8 µL X-tremeGENE™ HP DNA Transfection Reagent.
   b. Incubate transfection master mix for 15 min (20°C–25°C).
   c. Add 200 µL of the corresponding transfection master mix into the corresponding well of HEK 293T cells.
   d. Incubate cells 20–24 h at 37°C.
7. Day 3: Gently replace cell culture media with 3 mL of fresh DMEMc.
8. Day 4: Harvest virus-containing cell supernatant.
   a. Transfer cell supernatant into a 15 mL Falcon tube and remove cell-debris by centrifugation (2,000 × *g* for 10 min).
   b. Transfer the supernatant containing the viral stock into a new 15 mL Falcon tube and keep it at 4°C.
   c. Replenish the culture with 3 mL DMEMc.
9. Day 5: Harvest virus-containing cell supernatant.
   a. Transfer cell supernatant into a 15 mL Falcon tube and remove cell-debris by centrifugation (2,000 × *g* for 10 min).
   b. Combine the supernatant containing the viral stock with the viral stock harvested the day before.
   c. Aliquot the viral stocks in screw cap 1.5 mL sterile tubes and store the aliquots at −80°C. Aliquots of 100 or 50 µL are recommended to avoid several thaw-freeze cycles.

Note: A 2–7 × 10^4 $TCID_{50}$ (50% tissue culture infectious dose)/mL can be expected following this protocol. However, the infectivity of each viral stock can be variable, therefore titration of each viral stock should be performed as described in this protocol (target CD4+ T-cell generation section, step 5).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-CD3/8 bispecific antibody (1:2000)	NIH AIDS Reagent Program	ARP-12277
FITC Mouse Anti-HIV-1 core antigen, Clone KC57 (1:250)	Beckman Coulter	6604665
Purified Mouse Anti-Human CD28, Clone L293 (1:500)	BD Biosciences	348040
Purified Mouse Anti-Human CD49d, Clone L25 (1:1000)	BD Biosciences	340976

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
FITC Mouse Anti-Human CD107a, Clone H4A3 (1:45)	BD Biosciences	555800
FITC Mouse Anti-Human CD107b, Clone H4B4 (1:45)	BD Biosciences	555804
BUV496 Mouse Anti-Human CD3, Clone UCHT1 (1:100)	BD Biosciences	612940
Brilliant Violet 570™ Anti-Human CD4, Clone RPA-T4 (1:100)	BioLegend	300534
PerCP-Cy™5.5 Mouse Anti-Human CD8, Clone RPA-T8 (1:100)	BD Biosciences	560662
Chemicals, peptides, and recombinant proteins		
Potential T-cell epitope peptide panels: Pol	NIH AIDS Reagent Program	Cat#12961
Potential T-cell epitope peptide panels: Gag	NIH AIDS Reagent Program	Cat #12437
Efavirenz	Sigma-Aldrich	SML0536
Raltegravir	Sigma-Aldrich	CDS023737
Enfuvirtide	Sigma-Aldrich	SML0934
Polybrene (Hexadimethrine bromide)	Sigma-Aldrich	H9268
LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit	Thermo Fisher Scientific	L34957
LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit	Thermo Fisher Scientific	L34976
TrypLE™ Express Enzyme (1×), no phenol red	Thermo Fisher Scientific	12604021
CellTrace™ Far Red	Thermo Fisher Scientific	C34572
Phorbol-12-myristate-13-acetate (PMA)	Sigma-Aldrich	P1585-1MG
Ionomycin calcium salt	Sigma-Aldrich	I3909-1ML
BD GolgiStop™ (Containing Monensin)	BD Biosciences	554724
RPMI-1640	LONZA	12-167F
DMEM	LONZA	12-614F
AIM V™ Medium	Thermo Fisher Scientific	12055091
Opti-MEM I Reduced Serum Media	Thermo Fisher Scientific	31985062
Penicillin-Streptomycin (100×)	Thermo Fisher Scientific	15070063
GlutaMAX (100×)	Thermo Fisher Scientific	35050061
Fetal Bovine Serum (FBS)	Thermo Fisher Scientific	10099141
X-tremeGENE™ HP DNA Transfection Reagent	Merck-Roche	XTGHP-RO
Human IL-2 IS	Miltenyi Biotec	130-097-743
Human IL-7	Miltenyi Biotec	130-095-367
Human IL-15	Miltenyi Biotec	130-093-955
Paraformaldehyde (PFA) 16%, aqueous solutions	EMS	15710
Trypan Blue solution, 0.4%	Thermo Fisher Scientific	15250061
DPBS, no calcium, no magnesium	Thermo Fisher Scientific	14190144
Dimethylsulphoxide (DMSO), sterile	Sigma-Aldrich	D2650
Critical commercial assays		
Human CD8+ T-cell Isolation Kit	Miltenyi Biotec	130-096-495
Human CD4+ T-cell Isolation Kit	Miltenyi Biotec	130-096-533
MACS® MultiStand	Miltenyi Biotec	130-042-303
MiniMACS™ Separator	Miltenyi Biotec	130-042-102
MiniMACS™ Separation columns, type MS	Miltenyi Biotec	130-042-201
Fixation/Permeablization Kit	BD Biosciences	554717
NucleoBond Xtra Midi kit	MACHEREY-NAGEL	740410.50
Experimental models: Cell lines		
HEK-293-T Cells	Sigma-Aldrich	12022001VL
Recombinant DNA		
Plasmid: HIV-1 NL4-3^{AD8ENV} (HIV^{WT})	NIH AIDS Reagent Program	ARP-11346

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Plasmid: HIV^{NEF-ve} ($HIV^{\Delta Nef}$)	Dr Stuart G. Turville.[4]	N/A
Plasmid: pHEF-VSVg	NIH AIDS Reagent Program	ARP-4693
Software and algorithms		
FlowJo™ v10.8	BD	N/A
FACSDiva 8.0.3	BD Biosciences	N/A
Other		
Anti-Mouse Ig, κ/Negative Control Compensation Particles Set	BD Biosciences	552843
ArC™ Amine Reactive Compensation Bead Kit (for use with LIVE/DEAD™)	Thermo Fisher Scientific	A10628
Falcon® 96-well Round Bottom Cell Culture Microplate	CORNING	353077
Falcon® Round-Bottom Polystyrene FACS Tubes, 5 mL	CORNING	352054
Falcon® 25 cm^2 Cell Culture Flask with Vented Cap	CORNING	353108
MAX Efficiency™ Stbl2™ Competent Cells	Sigma-Aldrich	10268019
Conical 1.5 mL Tubes, with Clear Screw Caps, Sterile	Genesee Scientific	076-21-257
LSRFortessa Flow Cytometer	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

Flow cytometer

A LSRFortessa Flow Cytometer was used to develop the viral competition assay. This cytometer is equipped with 5 lasers and can detect 20 parameters as described in the table below:

LSRFortessa laser configuration				
Laser (nm)	Detector	Band pass filter	Dichroic mirror	Fluorophore/dye used
488	FSC	488/10	N/A	
	SSC	488/10	N/A	
	B530_30B	530/30	505LP	FITC
	B710_50A	710/50	685LP	PerCP-Cy5.5
403	V440_40F	440/40	N/A	
	V525_50E	525/50	505LP	LD Aqua
	V575_26D	575/26	550LP	BV570
	V660_20C	660/20	630LP	
	V710_50B	710/50	685LP	
	V780_60A	780/60	750LP	
355	U379_28C	379/28	N/A	
	U515_30B	515/30	450LP	BUV496
	U740_35A	740_35	690LP	
639	R670_14C	670/14	N/A	CTFR
	R730_45B	730/45	690LP	
	R780_60A	780/60	750LP	LD NIR
561	Y586_15D	586/15	570LP	
	Y610_20C	610/20	600LP	
	Y710_50B	710/50	685LP	
	Y780_60A	780/60	750LP	

The data acquisition was performed using the cytometry software BD FACSDiva 8.0.3.
FlowJo™ v10.8 was used for data analysis.

DMEMc media		
Reagent	Final concentration	Amount
DMEM	N/A	44 mL
Fetal Bovine Serum (FBS)	10% (v/v)	5 mL
100× Penicillin/Streptomycin	1% (v/v)	500 µL
100× GlutaMAX	1% (v/v)	500 µL
Total	**N/A**	**50 mL**

Store at 4°C for no longer than a month. Discard if media turns bright pink due to alkalinization caused by aeration.

RF10 media		
Reagent	Final concentration	Amount
RPMI-1640	N/A	44 mL
Fetal Bovine Serum (FBS)	10% (v/v)	5 mL
100× Penicillin/Streptomycin	1% (v/v)	500 µL
100× GlutaMAX	1% (v/v)	500 µL
Total	**N/A**	**50 mL**

Store at 4°C for no longer than a month. Discard if media turns bright pink due to alkalinization caused by aeration.

MACS wash Buffer		
Reagent	Final concentration	Amount
D-PBS	N/A	489.5 mL
Fetal Bovine Serum (FBS)	2% (v/v)	10 mL
EDTA	2 mM	500 µL
Total	**N/A**	**500 mL**

Store at 4°C for no longer than a month.

- **ARV stocks:** The anti-retrovirals (ARVs) Efavirenz, Enfuvirtide and Raltegravir can be resuspended in sterile DMSO within a laminar flow hood to prevent contamination. Resuspend each ARV individually at the following concentrations: Efavirenz 100 mM, Enfuvirtide 1 mM, Raltegravir 4 mM.

Aliquots of 10 µL are recommended to avoid several freeze-thaw cycles.

Store all ARVs at −20°C until needed.

10× ARV cocktail		
Reagent	Final concentration	Amount
RF10	N/A	988.25 µL
Efavirenz (1 mM)	1 µM	1 µL
Enfuvirtide (1 mM)	10 µM	10 µL
Raltegravir (4 mM)	3 µM	0.75 µL
Total	**N/A**	**1 mL**

Stock concentration of Efavirenz is 100 mM, therefore 1:100 dilution in RF10 is recommended prior making the 10× ARV cocktail.

The 10× ARV cocktail must be made immediately before use. Discard any left-over solution.

- Peptide pool reconstitution: Reconstitute lyophilized Potential T-cell Epitope (PTE) peptide pools corresponding to Gag and Pol HIV-1 proteins in dimethyl sulfoxide (DMSO) at 40 mg/mL.

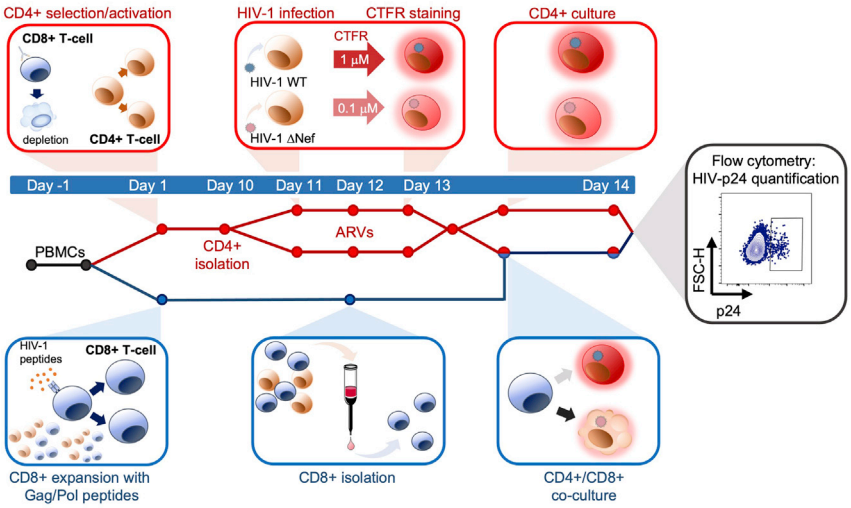

Figure 1. Schematic of the viral competition assay protocol

Note: Reconstituted peptide pools can be stored in aliquots of 10 μL. Additionally, we also recommend making several subaliquots in D-PBS at 4 mg/mL to optimize the use of the peptides and avoid several freeze-thaw cycles. Storing a total of 20 subaliquots containing 5 μL is recommended.

Note: Use sterile screw cap tubes to avoid peptide oxidation and exposition to moisture.

Note: Avoid re-using the same aliquot more than twice.

Store all the aliquots at −80°C.

- **1:1000 LIVE/DEAD Fixable Dead Cell Stain:** Add 1 μL of either Aqua or Near-IR (NIR) Fixable Dead Cell Stain to 999 μL D-PBS.

  Note: 1:1000 LIVE/DEAD staining solution must be made immediately before use. Discard any left-over solution.

- **8 mg/mL Polybrene:** Dissolve Polybrene at a concentration of 8 mg/mL in Milli-Q ultra-pure water and sterilize the solution by passing it through a 0.22-μm filter. Store small aliquots (20 μL) at −20°C until needed. Discard aliquots after use.
- **4% PFA solution:** Dilute 1 mL of 16% PFA with 3 mL of D-PBS. 4% PFA can be stored at 4°C away from light for up to 2 weeks.

⚠ CRITICAL: 16% PFA is classified as hazardous under Workplace Hazardous Materials Information System (WHIMIS) 2015. Handle inside a fume hood and use double gloves. Immediately rinse with water if there is contact with the skin. Dispose in appropriate chemical waste container.

STEP-BY-STEP METHOD DETAILS

This protocol describes the necessary steps to generate target HIV-1-infected CD4+ T-cells and effector HIV-1-specific CD8+ T-cells derived from PBMCs of individuals infected with HIV-1 (Figure 1).

PBMCs from HIV-1-infected individuals are necessary since memory HIV-1-specific CD8+ T-cells are required to expand enough HIV-1-specific effector CD8+ T-cells.

Target CD4+ T-cell generation

Timing: 10 days

This step involves the activation, selection, and expansion of target PBMCs-derived CD4+ T cells. These target cells should be prepared concurrently with the section effector HIV-1-specific CD8+ T-cell generation.

1. Day -1: Thaw PBMCs from HIV1-infected donors.

   Note: PBMCs from HIV-1-infected donors are necessary since the protocol requires the expansion of enough HIV-1-specific memory CD8+ T-cells enable to recognize and eliminate HIV-1-infected cells *in vitro*. Although a few naïve CD8+ T-cells from uninfected donors might potentially recognize some HIV-1-derived antigens, the number of cells would be too low and not enough to perform the viral competition assay.

   Note: Usually, 1×10^7 cells are cryopreserved per vial. Although cell recovery can be variable between donors, only 1×10^6 is required for the generation of target CD4+ T cells. All the remaining cells will be used for effector HIV-1-specific CD8+ T-cell generation section. The number of cells obtained from one cryovial containing 1×10^7 cells is usually enough to perform the viral competition assay, however please see troubleshooting 1 if cell recovery is too low.

   a. Warm a vial containing cryopreserved PBMCs in a 37°C water-bath.
   b. As soon as there is only a small piece of ice remaining, transfer 1 mL of thawed PBMCs into a 15 mL Falcon tube containing 9 mL of fresh prewarmed RF10.

   Note: Cryopreserved cells are usually preserved in freezing medium containing 10% DMSO to prevent the formation of ice crystals during the freezing process. However, DMSO has low toxicity to cells, therefore cells need to be washed as soon as possible after thawing. If a volume higher than 1 mL is thawed, scale up the necessary volume of RF10 to maintain a 1:10 dilution of the defrosted cells.

   c. Pellet the cells by centrifugation at 300 × *g* for 10 min. Remove the supernatant and resuspend the pellet in 10 mL of fresh RF10.
   d. Transfer the cells into a 25 mm culture flask and incubate them 12–18 h at 37°C.
2. Day 1: CD4+ T-cell activation, selection, and expansion.
   a. Perform cell counting and take 1×10^6 of PBMCs.
   b. Culture the cells in RF10 at a concentration of 1×10^6 cells/mL in a 24 well plate.
   c. Add the CD3/8 bispecific antibody to the cultured cells at a final concentration of 0.5 μg/mL.
   d. Supplement the culture with 100 IU/mL IL-2.

   Alternatives: The CD3/8 bispecific antibody can be added simultaneously with the IL-2 if they are previously premixed.

3. Day 3–10: Replace the media every 48–72 h with fresh RF10 supplemented with 100 IU/mL IL-2.

   Note: After 48–72 h of cell culture, cells should have settled on the bottom of the well. Therefore, media can be replaced as follows:

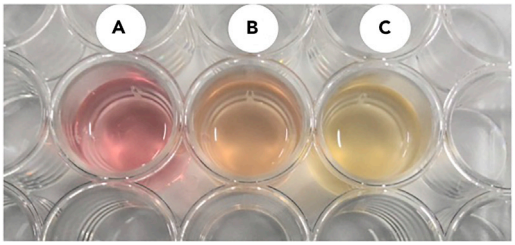

Figure 2. Replacement of cell culture media
(A–C) Picture illustrating color of fresh medium after replacement (A), culture medium after 2 days of cell culture (B) and culture medium from an overgrown cell culture (C). If the color of the cell medium is similar to (C), the medium must be immediately replaced.

a. Without disturbing cells from the bottom of the well, remove approximately 900 μL of the cell culture medium.
b. Add 900 μL of fresh, IL-2-suppplemented, RF10. Take care not to disturb the cells from the bottom of the well.

Note: Addition of the CD3/8 bispecific antibody is not necessary when replacing the media.

Note: Whether the media need to be replaced or not may be judged based on the media color. Please see Figure 2 for more details.

Note: CD3/8 bispecific antibody induces fast cell proliferation. Cells should be split into multiple wells if necessary. As shown in Figure 3, stimulated cells form cell clusters and may cover the bottom of the well. Please see Figure 3 for more details regarding when cells should be split.

Note: PBMC treatment with CD3/8 bispecific antibody results in the elimination of CD8+ T-cells and enrichment of activated CD4+ T-cells.[1,2]

⚠ CRITICAL: This protocol involves the manipulation of HIV-1 stocks and samples from individuals infected with HIV-1, therefore all the lab work must be undertaken in Biosafety Level 3 (BSL-3) laboratories.

HIV-1 infection of target CD4+ T-cells

Timing: 3 days

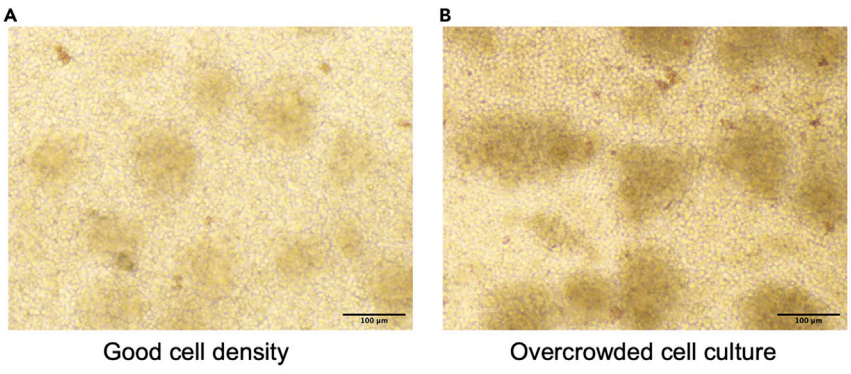

Figure 3. Cell culture density
Microscopy images depicting cell culture of PBMCs after stimulation with anti CD3/28 bispecific antibody.
(A) Cells culture showing good cell density.
(B) Cells from an overcrowded culture. Split cells when convenient to avoid cell density as shown in (B).
Scale bars represent 100 μm.

In this step, the expanded CD4+ T-cells will be isolated and infected with HIV^{WT} or $HIV^{\Delta Nef}$ to be cocultured with effector HIV-1 specific CD8+ T-cells in viral competition assay section. Cells will also be stained with the fluorescent dye CellTrace™ Far Red (CTFR) to identify each target population by flow cytometry.

Note: Although CD3/8 bispecific antibody treatment results in CD4+ T-cell enrichment (approximately 70% CD4+ T-cells), isolation of CD4+ T-cells by magnetic bead selection is necessary to achieve >90% purity.

4. Day 10: CD4+ T-cell isolation.
   a. At day 10 of cell culture, pool cells from every well and count total cell number.

   Note: Cell number can be variable between donors. However, approximately 4×10^6 cells can be expected after the treatment with CD3/8 bispecific antibody. Determining the number of cells is necessary to calculate the volume of reagents to be used in the CD4+ T-cell isolation step (below).

   b. Spin cells down at 300 × *g* for 5 min.
   c. Perform CD4+ T-cell purification by using a CD4+ T-cell isolation kit (see key resources table) as follows:
      i. Resuspend cell pellet in 40 µL of MACS wash buffer per 1×10^7 total cells.

   Note: When working with fewer cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly.

      ii. Add 10 µL of CD4+ T Cell Biotin-Antibody Cocktail (from the CD4+ T-cell isolation kit) per 1×10^7 total cells.
      iii. Mix well and incubate for 5 min in the refrigerator (2°C–8°C).
      iv. Add 30 µL of MACS wash buffer per 1×10^7 total cells.
      v. Add 20 µL of CD4+ T Cell MicroBead Cocktail (from the CD4+ T-cell isolation kit) per 1×10^7 total cells.
      vi. Mix well and incubate for 10 min in the refrigerator (2°C–8°C).
      vii. A minimum of 500 µL of cell suspension is required for magnetic separation. If necessary, add MACS buffer to the cell suspension.
      viii. Set the miniMACS separator column on the miniMACS separator (see key resources table) as shown in Figure 4.

   Note: Always wait until the column reservoir is empty before proceeding to the next step.

      ix. Prepare column by rinsing with 500 µL of MACS wash buffer.
      x. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells in a sterile FACS tube, representing the enriched CD4+ T-cells.
      xi. Wash column with 500 µL of MACS wash buffer twice. Collect unlabeled cells that pass through, representing the enriched CD4+ T-cells, and combine with the effluent from step 4.c.x.
   d. Plate isolated CD4+ T-cells in a 96 well plate at a concentration of 1×10^5 cells /100 µL RF10 (supplemented with 100 IU/mL IL-2) per well.

   Note: Pure CD4+ T-cell yield is typically around 50%–90% of the number of cells counted in step 4.a.

   e. Incubate the cells 12–18 h at 37°C.
5. Day 11: HIV-1 infection of target CD4+ T-cells.

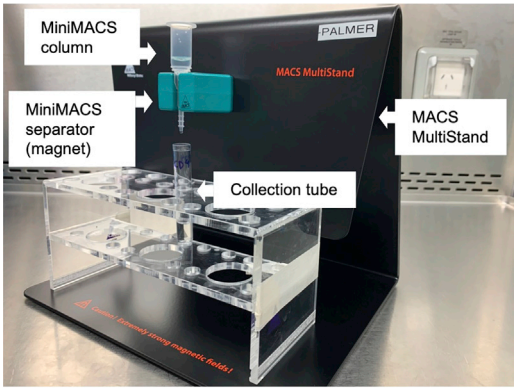

Figure 4. Isolation kit set
Photo showing different components of the T-cell Isolation kit.

Note: In this step, isolated CD4+ T-cells are infected with the corresponding viral stock previously made (see preparation of viral stocks). Since the viral titer of each stock and the susceptibility to HIV-1 infection between donors varies, we recommend performing a titration of each viral stock as indicated below (Figure 5A). This should be performed in every experiment using cells from different donors.

The titration performed in this step is also necessary to select cells infected with both viruses at a similar level. This will be important when conducting the viral competition assay.

Note: Infection of primary CD4+ T-cells with HIV-1 can be challenging. To overcome this challenge, we recommend using polybrene. Polybrene is frequently used to increase viral infection or lentiviral transduction.[5]

a. Thaw the viral stocks by incubating one aliquot of each stock at 37°C in a water bath.

Note: Defrosting the viral stocks at 37°C prevents ice crystal formation.

b. Add to the corresponding wells 2.5, 5, 10 and 20 μL of each viral stock in duplicate (Figure 5A).
c. One or two wells with cells should remain uninfected as mock control (Figure 5A). This control will be necessary for the cytometry analysis (Figures 5B and 5C).
d. Perform viral infection by spinoculation:
   i. Add 0.8 μg of polybrene (final concentration: 8 μg/mL) to each well.

Note: Polybrene stock concentration is 8 mg/mL, therefore a dilution using RF10 can be performed prior its addition to the cell culture.

   ii. Centrifugate the plate at 800 × *g* for 90 min at approximately 25°C.
   iii. Replace the media with fresh 100 μL of RF10 supplemented with 100 IU/mL IL-2.
e. Incubate cells 12–18 h at 37°C.

Note: Since Nef (and most of the HIV-1 accessory proteins) contributes to viral replication,[6] it is necessary to avoid viral spread to ensure only one round of infection occurs by adding antiretrovirals to prevent viral spread. This is important to obtain similar levels of infection between cells infected with both viruses.

f. Add antiretrovirals (ARVs) to the culture media with infected cells. Final concentration: 100 nM Efavirenz, 1 μM Enfuvirtide and 300 nM Raltegravir.

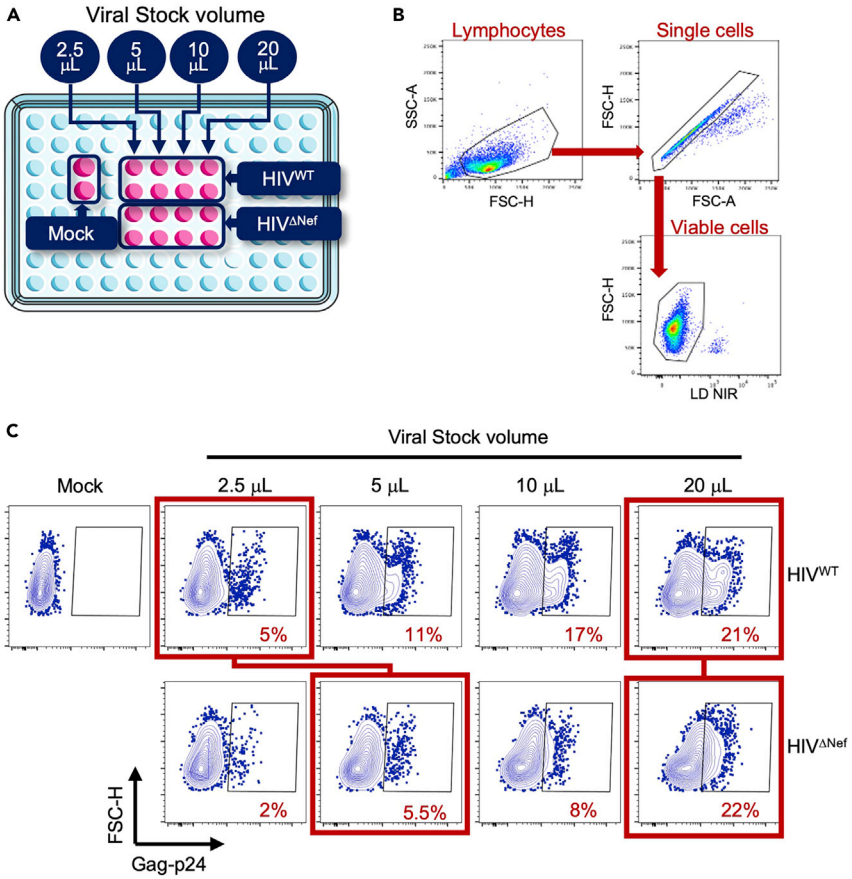

Figure 5. Viral stock titration

(A) Schematic of recommended plating strategy.

(B) Representative cytometry showing gating strategy to select lymphocyte, single and viable cells.

(C) Representative cytometry showing the proportion of Gag-p24 positive cells and selection of cells infected at similar level.

Note: We recommend preparing a 10× cocktail with ARVs. Then add 10 µL of the ARV cocktail to each well containing HIV-1-infected cells.

6. Day 13: Quantification of infection level by HIV-1 Gag-p24 intracellular staining.
   a. Pool duplicate wells and transfer the cells to a 1.5 mL Eppendorf tube.
   b. Take 50 µL of cells from each tube and incubate remaining cells at 37°C.
   c. Determine the level of infection by staining of the HIV-1 protein Gag-p24 as follows:

Note: Also include uninfected CD4+ T-cells to set up the flow cytometry gating strategy as shown in Figures 5B and 5C.

Note: Every cell wash implies removing the supernatant after centrifuging each tube at 300 × *g* for 5 min and resuspending the cells in the designated buffer.

   i. Wash cells with 100 µL D-PBS once and stain with 100 µL of 1:1000 Live Dead Near Infra-Red (LDNIR).
   ii. Incubate the cells for 15 min at 4°C in the dark.
   iii. Wash cells with 100 µL D-PBS once.

iv. Fix cells with Cytofix/Cytoperm buffer (from Fixation/Permeabilization kit, see key resources table) for 15 min 4°C in the dark.
v. In the meantime, dilute the anti-Gag-p24 antibody (anti-HIV-1 core, clone KC57, see key resources table) 250 times: e.g., 3 μL antibody in 750 μL 1× Perm/Wash buffer (from Fixation/Permeabilization kit, see key resources table).
vi. Wash cells with 100 μL of 1× Perm/Wash buffer.
vii. Pellet the cells by centrifugation (300 × *g* for 5 min) and remove the supernatant.
viii. Resuspend cells in 100 μL of diluted anti-Gag-p24 to each tube.
ix. Incubate cells for 20 min at 4°C in the dark.
x. Wash cells once with 100 μL of 1× Perm/Wash buffer.
xi. Wash cells once with 100 μL of D-PBS.
xii. Resuspend the cells in 200 μL D-PBS and run the samples on a flow cytometer.

Note: Acquisition of 15–25,000 events is enough to quantify the proportion of HIV-1-infected cells by flow cytometry.

Note: Compensation controls can be prepared by using anti-Mouse Ig compensation beads (see key resources table) for antibody staining, and ArC amine reactive beads (see key resources table) for Live Dead staining, following manufacturers' instructions (anti-Mouse Ig compensation beads instructions; ArC amine reactive beads instructions). Same procedure can be applied for every cytometry indicated in the following sections.

Note: Fluorophores with low spill over between them have been chosen in order to set a correct fluorescence compensation. In addition, when the corresponding antibodies were titrated, the voltage values for each photomultiplier tube (PMT) were optimized to maintain an adequate visual separation between the positive and negative population.

Note: If low or undetectable level of Gag-p24 is observed, please see troubleshooting 2.

d. Once Gag-p24 has been quantified, select wells (duplicates in the plate) with similar level of infection (Figure 5C) to be stained with CellTrace Far Red (CTFR).

7. Day 13: CTFR staining of HIV-1 infected CD4+ T-cells.

Note: This step is performed to distinguish cells infected with either HIV^{WT} or $HIV^{\Delta Nef}$ in a co-culture system. Both populations of infected cells are stained with CTFR, but the concentration of the dye used to stain each group of infected cells is based on their viral infection (HIV^{WT} or $HIV^{\Delta Nef}$). Both populations can be easily visualized by flow cytometry (Figure 6).

Note: Every cell wash implies removing the supernatant after centrifuging each tube at 300 × *g* for 5 min and resuspending the cells in the designated buffer.

a. Based on the Gag-p24 quantification obtained in the previous step, select the wells containing cells with similar levels of infection for both viruses (Figure 5C).

Note: If after quantifying the proportion of Gag-p24 positive cells, similar level of infection between HIV^{WT} and $HIV^{\Delta Nef}$-infected cells is not observed, please see troubleshooting 3.

b. Transfer the selected cells into a 1.5 mL Eppendorf tube and perform CTFR staining:
i. Wash cells once with 200 μL D-PBS.
ii. Dilute the CTFR stock in D-PBS. Prepare one tube with 1 μM CTFR and another tube with 0.1 μM CTFR.

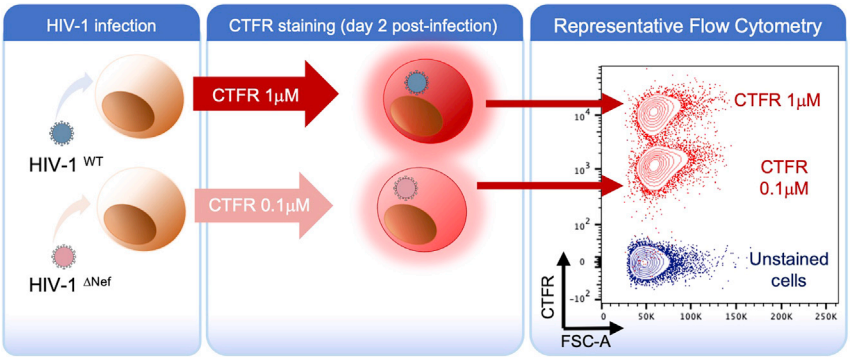

Figure 6. CTFR staining of HIV-1-infected CD4+ T-cells

Schematic of CD4+ T-cells infected with either HIV^{WT} or $HIV^{\Delta Nef}$ and stained with 1 μM or 0.1 μM CTFR, respectively. Representative cytometry of cells stained with CTFR is shown (right).

iii. Stain cells with 200 μL of 1 μM (cells infected with HIV^{WT}) or 200 μL of 0.1 μM CTFR (cells infected with $HIV^{\Delta Nef}$).
iv. Incubate cells for 15 min at 37°C in the dark.
v. Add 400 μL RF10 to each tube and spin cells down for 5 min at 300 × *g*.
vi. Remove the supernatant and resuspend the cells in 150 μL of fresh RF10.

Effector HIV-1-specific CD8+ T-cell generation

Timing: 12 days

This section describes the necessary steps to generate effector HIV-1-specific CD8+ T-cells by using peptides derived from the HIV-1 proteins Gag and Pol.

Note: This step is performed concurrently with the target CD4+ T-cell generation step.

Note: 80%–90% of the PBMCs thawed in the target CD4+ T-cell generation section can be used in this step.

8. Day 1: Peptide stimulation.
   a. Take the remaining PBMCs from the target CD4+ T-cell generation section, step-2a and incubate the cells at a concentration of 2–4 × 10^6 cells/100 μL in AIM-V media in a sterile FACS tube.

Note: FBS is thought to interfere with peptide binding. We therefore recommend using AIM-V serum-free media to pulse PBMCs with the HIV-1 peptides.

Note: Every cell wash implies removing the supernatant after centrifuging each tube at 300 × *g* for 5 min and resuspending the cells in the designated buffer or cell culture medium.

   b. Pulse PBMCs with 4 μg/mL Gag and Pol peptide pool 2 h at 37°C.
   c. Perform one wash with RF10.
   d. Resuspend the cells in RF10 containing 1 μg/mL of anti-CD28 antibody (see key resources table).

Note: Cell concentration should be 4 × 10^6 cells/mL.

   e. Culture the cells for 12–18 h at 37°C in a 24 well plate.

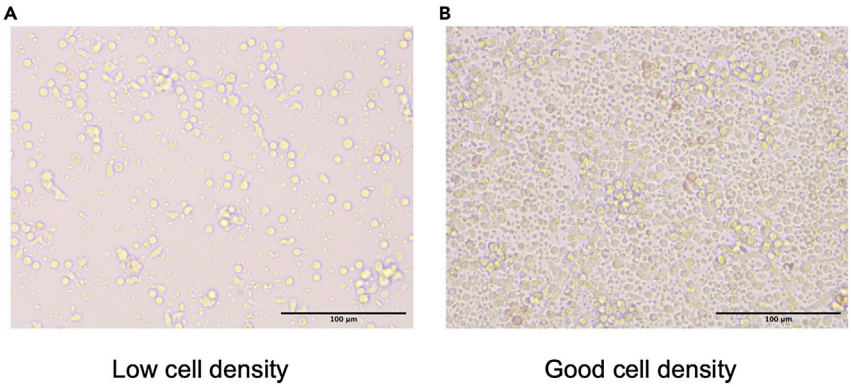

Figure 7. Density of PBMC culture

Representative microscopy images showing cell density after plating PMBCs post peptide stimulation.
(A) Cells cultured at a low concentration (1 × 10^{10} cells/mL).
(B) Cells cultured at the proper concentration (4 × 10^{10} cells/mL). Scale bars represent 100 µm.

Note: Up to 1 mL of the cell suspension can be plated per well in a 24 well plate.

Note: Cell density is very important in the first few days of CD8+ T cell expansion. If the number of cells is less than 4 × 10^6, cells can be plated in a 48 well plate. Of note, cells should cover the bottom of the well without large gaps between group of cells. Figure 7 illustrates cell cultures at low (less than 4 × 10^6 cells/mL) and adequate density (4 × 10^6 cells/mL).

9. Day 2: Supplement the cell culture with IL-2 (100 IU/mL), IL-7 (200 IU/mL) and IL-15 (200 IU/mL).
   a. Replace the media every 48–72 h with fresh media supplemented with IL-2, IL-7 and IL-15.

Note: Always supplement RF10 media with IL-2 (100 IU/mL), IL-7 (200 IU/mL) and IL-15 (200 IU/mL) when the addition of these 3 cytokines is indicated.

Note: After one week of culture, the rate of cell proliferation increases. If necessary, cells can be split into multiple wells. As shown in Figure 8, stimulated cells form cell clusters and may cover the bottom of the well. Avoid overcrowded cultures as illustrated in Figure 8.

Note: Whether the media need to be replaced may be judged based on the media color. Please see Figure 2 for more details.

Note: After 48–72 h of cell culture, cells should have settled on the bottom of the well. Therefore, media can be replaced by aspirating approximately 900 µL of the old medium, and adding 900 µL of fresh, IL-2-, IL-7-, IL-15-suppplemented RF10. Take care not to disturb the cells from the bottom of the well.

10. Day 11: Quantification of HIV-1 specific CD8+ T-cells (optional).

Note: This step is to verify and quantify the presence of HIV-1-specific CD8+ T-cells after the expansion by quantifying the expression of the degranulation markers CD107a/b upon peptide re-stimulation.

   a. Take 6 × 10^5 PBMCs and split them into 3 FACS tubes.

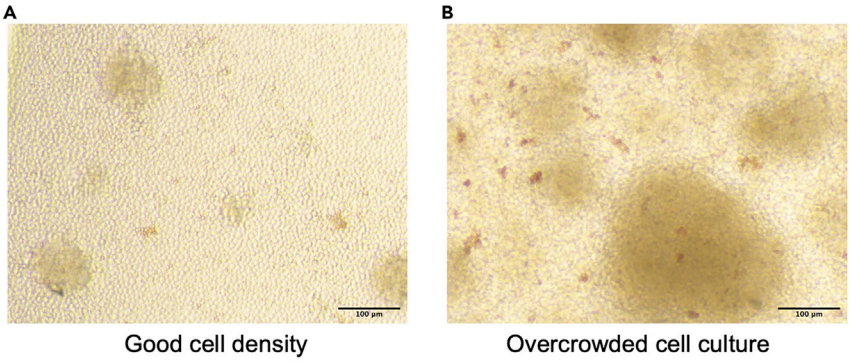

Figure 8. Cell culture density

Microscopy images depicting culture of PBMCs after stimulation with HIV-1 PTE peptide pool.
(A) Cells culture showing good cell density.
(B) Cells from an overcrowded culture. Split cells when convenient to avoid cell density as shown in (B). Scale bars represent 100 µm.

Note: Extra cells can be taken for Fluorescence Minus One (FMO) control. During the data analysis, FMO controls are recommended to set the gate and cutoff for CD107a/b positive cells, since unstimulated cells may express low levels of CD107a/b.

Note: Due to the small number of conditions and low number of cells, the stimulation and flow staining can be alternatively performed in a V-bottom 96 well plate.

Note: Every cell wash implies removing the supernatant after centrifuging each tube or plate at 300 × *g* for 5 min and resuspending the cells in the designated buffer.

b. Wash cells with RF10 once and resuspend them in 200 µL of RF10 media.
c. Make a master mix containing: 17.6 µL RF10, 1.6 µL of anti-CD28, 0.8 µL of anti-CD49d, 20 µL anti-CD107a FITC, and 20 µL anti-CD107b FITC antibodies.

Note: Addition of CD107a/b antibodies during the stimulation is necessary since the extracellular expression of CD107a/b is transient due to the recycling of the CD107a/b (+) granules.

d. Add 15 µL of master mix to each FACS tube or well if a plate is used.
e. Next, stimulate cells as follows:
   i. Tube 1: 4 µg/mL of Gag/Pol peptide pool.
   ii. Tube 2: 50 ng/mL PMA + 1 µM Ionomycin (positive control).
   iii. Tube 3: no stimulation (negative control).
f. Add the protein transport inhibitor GolgiStop (Monensin) at a final dilution of 1:1000 to each tube. Monensin treatment improves CD107a/b staining.
g. Incubate the cells for 5 h at 37°C.
h. Cell surface staining:
   i. Spin down cells by centrifugation at 300 × *g* for 5 min.
   ii. Wash the cells with 200 µL D-PBS once.
   iii. Remove the supernatant and stain cells with Live Dead NIFR for 15 min at 4°C in the dark.
   iv. Spin down cells by centrifugation at 300 × *g* for 5 min.
   v. Wash the cells with 200 µL D-PBS once.
   vi. Make a master mix containing 300 µL D-PBS/2% FBS, and 3 µL of anti-human CD3, CD4 and CD8 antibodies (see key resources table).

vii. Add 100 μL of the master mix to each tube and incubate cells for 20 min at 4°C in the dark.
viii. Spin down cells by centrifugation at 300 × *g* for 5 min.
ix. Wash the cells with 200 μL D-PBS/2% FBS once.
x. Resuspend cells in 200 μL D-PBS/2% FBS and run the tubes on a Flow Cytometer.

Alternatives: Cells can be fixed with 4% PFA if samples will not be run on the Flow Cytometer immediately.

i. Cell fixation:
   i. Spin down cells by centrifugation at 300 × *g* for 5 min.
   ii. Wash the cells with 100 μL D-PBS once.
   iii. Remove the supernatant and fix cells with 100 μL 4% PFA for 15 min at 4°C in the dark.
   iv. Spin down cells by centrifugation at 300 × *g* for 5 min.
   v. Wash the cells with 100 μL D-PBS once.
   vi. Resuspend cells in 200 μL D-PBS.

Note: Controls for fluorescent compensation need to be included.

Note: Fluorophores with low spill over between them have been chosen in order to set a correct fluorescence compensation. In addition, when the corresponding antibodies were titrated, the voltage values for each photomultiplier tube (PMT) were optimized to maintain an adequate visual separation between the positive and negative population.

Note: Usually the proportion of HIV-1-specific CD8+ T-cells is relatively low and donor dependent. We usually observed values of cells responding to peptide-restimulation between 1%–4%, however this is enough to perform the viral competition assay (Figure 9).

11. Day 12: CD8+ T-cell isolation.
    a. At day 12 of cell culture, pool cells from every well and count cell number.

Note: Cell number can be variable between donors, however, approximately 1 × 10^7 cells is expected. Determining the number of cells is necessary to calculate the volume of reagents needed for the CD8+ T-cell isolation step (below).

    b. Perform CD8+ T-cell purification by using a CD8+ T-cell isolation kit (see key resources table) as follows:
       i. Spin cells down for 5 min at 300 × *g*.
       ii. Resuspend cell pellet in 40 μL of MACS wash buffer per 1 × 10^7 total cells.

Note: When working with fewer cells, use the same volumes as indicated. When working with higher cell numbers, scale up all reagent volumes and total volumes accordingly.

       iii. Add 10 μL of CD8+ T Cell Biotin-Antibody Cocktail (from the CD8+ T-cell isolation kit) per 1 × 10^7 total cells.
       iv. Mix well and incubate for 5 min in the refrigerator (2°C–8°C).
       v. Add 30 μL of MACS wash buffer per 1 × 10^7 total cells.
       vi. Add 20 μL of CD8+ T Cell MicroBead Cocktail (from the CD8+ T-cell isolation kit) per 1 × 10^7 total cells.
       vii. Mix well and incubate for 10 min in the refrigerator (2°C–8°C).

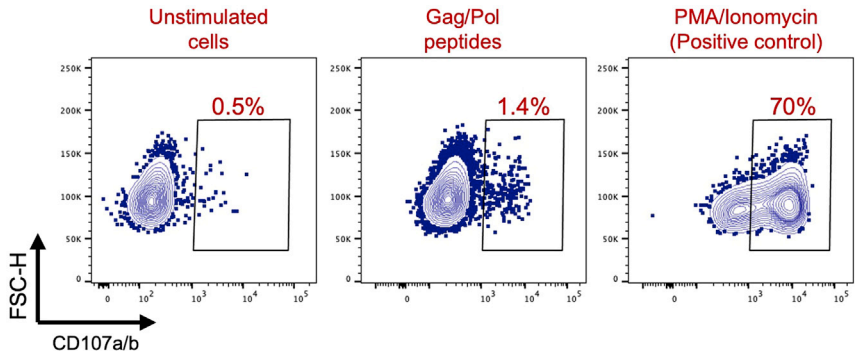

Figure 9. HIV-1-specific CD8+ T-cells

Representative cytometry of CD107a/b expression after 12 days of cell expansion. An increase in the expression of the degranulation marker CD107a/b upon the restimulation with Gag/Pol peptides indicates expansion of HIV-1-specific cells.

viii. A minimum of 500 μL of cell suspension is required for magnetic separation. If necessary, add MACS buffer to the cell suspension.

ix. Set the miniMACS separator column on the miniMACS separator (see key resources table) as shown in Figure 4.

Note: Always wait until the column reservoir is empty before proceeding to the next step.

x. Prepare column by rinsing with 500 μL of MACS wash buffer.

xi. Apply cell suspension onto the column. Collect flow-through containing unlabeled cells in a sterile FACS tube, representing the enriched CD8+ T-cells.

xii. Wash column with 500 μL of MACS wash buffer twice. Collect unlabeled cells that pass through, representing the enriched CD8+ T-cells, and combine with the effluent from step 11.b.xi.

c. After isolation, cells can be cultured in a sterile FACS tube at 1–2 × 10^6 cells / mL in RF10 supplemented with IL-2, IL-7 and IL-15.

d. Incubate the cells for 12–18 h at 37°C.

Note: CD8+ T-cell recovery after isolation can be variable between donors (35%–70% of the expanded PBMCs). See troubleshooting 4 if cell recovery is too low.

Viral competition assay

Timing: 2 days

This protocol evidences the contribution of the viral protein Nef in protecting HIV-1-infected cells from CD8+ T-cell-mediated clearance. In this section cells generated from previous steps are combined.

12. Day 13: Prepare target CD4+ T-cells.

a. Count the cell number of HIV^{WT}- and $HIV^{\Delta Nef}$-infected/CTFR-stained CD4+ T-cells from the HIV-1 infection of Target CD4+ T-cells section- step 6b.

b. Combine 1 × 10^5 cells of both HIV^{WT}- and $HIV^{\Delta Nef}$-infected CD4+ T-cells in a 1.5 mL Eppendorf tube.

c. Spin cells down by centrifugation at 300 × *g* for 5 min.

d. Remove the supernatant and resuspend cells in 200 μL RF10.

e. Split 100 μL of mixed cells into 2 wells in a 96 U-bottom well plate. Effector CD8+ T-cells will be added to one of these wells.

13. Day 13: Prepare effector CD8+ T-cells and co-culture.
    a. Count cell number of isolated CD8+ T-cells from the effector HIV-1-specific CD8+ T-cell generation section step-11d.
    b. Spin cells down by centrifugation at 300 × *g* for 5 min.
    c. Remove the supernatant and resuspend cells in RF10. Final concentration should be 3×10^5 cells/100 μL RF10.
    d. Add 100 μL of effector CD8+ T-cells to the corresponding well containing target CD4+ T-cells. This well will correspond to the CD4 (target):CD8 (effector) co-culture condition.
    e. Supplement the culture media with 20 IU/mL IL-2. At this stage proliferation of either CD8+ or CD4+ T-cells is no longer required. This concentration of IL-2 is enough to promote survival of activated T-cells without promoting excessive cell proliferation.
    f. The cell cultures should be also supplemented with ARVs as described in HIV-1 infection of Target CD4+ T-cells, step 5f.
    g. Incubate cells 12–18 h at 37°C.

Note: All the recommended volumes and number of cells can be scaled up, as long as the concentration of cells and the effector:target (3:1) ratio remain constant.

14. Day 14: Quantification of CD8+ T-cell-mediated clearance of HIV-1 infected-cell.

Note: Every cell wash implies removing the supernatant after centrifuging each tube at 300 × *g* for 5 min and resuspending the cells in the designated buffer.

    a. Transfer cells into FACS tubes.
    b. Perform cell surface and intracellular Gag-p24 staining:
        i. Wash cells with 200 μL D-PBS once and stain with 100 μL 1:1000 Live Dead Aqua for 15 min at 4°C in the dark.
        ii. Wash cells with 200 μL D-PBS once.
        iii. Make a master mix containing 300 μL D-PBS/2% FBS and 3 μL of anti-human CD8 antibody (see key resources table).
        iv. Add 100 μL of the master mix to each tube and incubate cells for 20 min at 4°C in the dark.
        v. Spin cells down by centrifugation at 300 × *g* 5 min.
        vi. Wash the cells with 200 μL D-PBS/2% FBS once.
        vii. Fix cells with 200 μL Cytofix/Cytoperm buffer (from Fixation/Permeabilization kit, see key resources table) for 15 min at 4°C in the dark.
        viii. In the meantime, dilute the anti-Gag-p24 antibody 250 times: e.g., 3 μL antibody in 750 μL 1× Perm/Wash buffer (from Fixation/Permeabilization kit, see key resources table).
        ix. Wash cells with 200 μL 1× Perm/Wash buffer.
        x. Pellet the cells by centrifugation and remove the supernatant.
        xi. Add 100 μL of diluted anti-Gag-p24 to each tube.
        xii. Incubate cells for 20 min at 4°C in the dark.
        xiii. Wash cells once with 200 μL 1× Perm/Wash buffer.
        xiv. Wash cells once with 200 μL D-PBS.
        xv. Resuspend the cells in 200 μL D-PBS and run the samples through a flow cytometer.

Note: Approximately 50–70,000 events can be recorded from the tube containing CD4+ T-cells only, and 100–150,000 events can be recorded from the tube containing co-cultured CD8+ and CD4+ T-cells.

Note: Controls for fluorescent compensation need to be added.

Note: Fluorophores with low spill over between them have been chosen in order to set a correct fluorescence compensation. In addition, when the corresponding antibodies were titrated, the voltage values for each photomultiplier tube (PMT) were optimized to maintain an adequate visual separation between the positive and negative population.

Alternatives: Flow staining can alternatively be performed in V-bottom 96 well plates.

Alternatives: Different time points can be added to this protocol. For instance, intracellular Gag-p24 values can also be assessed after 6 h of co-culture. This would allow for the assessment of the kinetics of the differential killing of cells infected with the different viruses.

Optional: In our study we used some of the co-cultured cells to perform full-length proviral sequencing and characterize the HIV-1-infected cells which survived to the CD8+ T-cell-mediated clearance.[1] Details and extra steps needed to perform this sequencing are detailed in Duette et al.[1]

EXPECTED OUTCOMES

This method has been developed to evaluate and quantify the contribution of the viral protein Nef in protecting HIV-1-infected cells from CD8+ T-cell-mediated clearance. At the end of this assay, it is expected that differences in intracellular p24 values will be present when comparing cells infected with HIV-1 (HIV^{WT}) with cells infected with Nef-deleted HIV-1 ($HIV^{\Delta Nef}$). Target CD4+ T-cells infected with $HIV^{\Delta Nef}$ are more susceptible to cytotoxic CD8+ T-cell activity since MHC-I downmodulation caused by Nef is prevented and antigen presentation is enhanced.[7,8] A representative experiment showing expected differences can be seen in Figure 10.

CD8+ T-cell cytotoxic capacity can be variable between donors, however the effector:target ratio recommended in this protocol has consistently shown the expected outcomes. Nevertheless, if the results are obscured by excessive CD8+ T-cell-mediated clearance of the target cells, please see troubleshooting 5.

QUANTIFICATION AND STATISTICAL ANALYSIS

To show the preferential CD8+ T-cell-mediated clearance of cells infected with $HIV^{\Delta Nef}$, relative p24 values can be calculated as follows:

15. Proportion of p24 positive cells of the culture control (CD4+ T-cells only) is considered 100%. This is calculated for HIV^{WT}- and $HIV^{\Delta Nef}$-infected cells separately. In the example shown in Figure 11, 4.38% and 4.71% are normalized to 100% of p24 level. Then, the relative proportion of p24 positive cells in the CD8+/CD4+ T-cell co-culture is calculated. For example, 1.37% cells infected with HIV^{WT}, after the co-culture with effector CD8+ T-cells, represents 31.27% when 4.38% is normalized to 100%. In the case of the cells infected with $HIV^{\Delta Nef}$, 0.33% represents 7% when 4.71% is normalized to 100% (Figures 11A and 11B).

Note: To calculate standard deviation and significant statistical differences multiple donors need to be assessed. We recommend a minimum of 3 donors, however this can vary and is dependent on the results obtained.

LIMITATIONS

The viral competition assay described in this protocol has some limitations. Although this method clearly shows the reduction of CD8 T-cell-mediated clearance of cells infected with HIV-1 containing the viral protein Nef, the approach is carried out in an *in vitro* system. There are many other factors

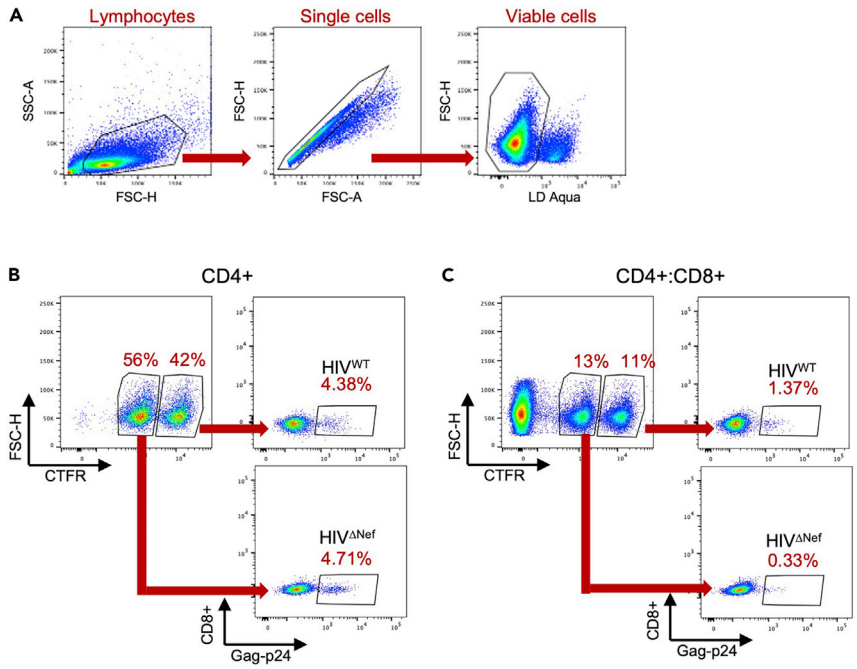

Figure 10. Viral competition assay

Representative flow cytometry representing the proportion of Gag-p24 positive cells for cells infected with either HIV^{WT} or $HIV^{\Delta Nef}$.

(A) Representative cytometry showing gating strategy to select lymphocyte, single and viable cells.

(B) Culture control of infected CD4+ T-cells.

(C) CD8+ T-cell and infected CD4+ T-cell co-culture.

Adapted from Duette et al.[1]

that can also contribute to immune escape *in vivo*, such as CTL escape mutations or other viral proteins.[9,10] However, different viral constructs can be designed to harbor CTL escape mutations or deletions for different HIV-1 proteins in order to test their role in immune escape by applying this viral competition assay.

A second limitation is that the generation of HIV-1-specific memory CD8+ T-cells is limited to the pool of peptides used in this protocol. This would not cover the entire spectrum of HIV-1-specific memory CD8+ T-cells found in every infected individual. However, it is very well known that Gag and Pol represent the most immunogenic HIV-1 proteins,[11] allowing for the expansion of enough

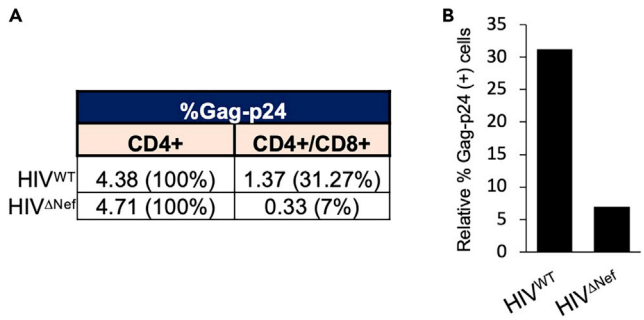

Figure 11. Quantification of relative proportion of HIV-1 infected cells after performing the viral competition assay

(A) Table showing p24 values obtained in a representative experiment.

(B) Bar graph showing the proportion of p24 positive cells relative to the culture control (CD4+ T-cells only) after performing the viral competition assay.

HIV-1-specific memory CD8+ T-cells to show CD8+ T-cell-mediated clearance. Moreover, the Potential T-cell Epitope (PTE) peptide panels used in this protocol, are designed to represent the most frequent potential T-cell epitopes from the viral protein Gag and Pol embedded in the sequence of circulating strains of HIV-1 worldwide.[12]

Another limitation is the access to samples from individuals living with HIV-1. The PBMCs used in our study were obtained from a specific cohort of individuals living with HIV-1.[1] Samples obtained from different cohorts of HIV-1-infected participants may result in different outcomes in terms of the efficiency of the CD8+ T-cell-mediated clearance. However, a similar methodology[2] revealed similar levels of CD8+ T-cell-mediated clearance using a different cohort of HIV-1-infected participants.

TROUBLESHOOTING

Problem 1

Low cell recovery and/or clump formation after thawing cryopreserved PBMCs from HIV-1-infected individuals.

Potential solution

Cell clumping is a common problem following thawing of PBMCs isolated from blood and may lead to loss of cells or affect cell function or phenotype. The incorporation of a DNase treatment step in the standard thawing procedure efficiently avoids clump formation, improving cell recovery.[13]

Problem 2

Low infectivity of the HIV-1-viral stocks produced in HEK 293T cells.

Potential solution

If cells infected with the HIV-1-viral stocks produced in HEK 293T cells show low or undetectable levels of infection, we recommend the following:

- Use fresh HEK 293T cells every time that viral stock is produced.
- Efficiency of transfection can be tested by Gag-p24 staining of the transfected cells.
- Mycoplasma contamination should also be routinely tested for since contamination with mycoplasma can impact cell proliferation and transfection efficiency.
- Viral stock infectivity should not significantly decrease after a few years if stocks are properly stored at −80°C. However, if old stocks have been used, showing low infectivity, we suggest making new viral stocks.

Problem 3

Titration of HIV^{WT} and $HIV^{\Delta Nef}$ stocks does not show similar levels of Gag-p24 in HIV-1-infected CD4+ T-cells.

Potential solution

The volume of the viral stock used to titrate the different HIV-1 stocks indicated in this protocol were based on our own experience and preliminary results from our lab. However, different volumes can be tested if the volumes suggested in this protocol do not show cells infected with HIV^{WT} or $HIV^{\Delta Nef}$ at similar levels.

Problem 4

Low number of CD8+ T-cells after isolation.

Potential solution

If the initial number of PBMCs used to expand HIV-1-specfic CD8+ T-cells is low, the number of CD8+ T-cells after the isolation step can be low as well. Although not having enough cells to perform

the viral competition assay is unlikely, the number of cells per condition can be scaled down as long as the effector:target (3:1) ratio remains constant.

Problem 5

Excessive CD8+ T-cell-mediated clearance of target cells obscures differences in the proportion of HIV-1-infected cells.

Potential solution

This method relies on the capacity to detect preferential clearance of cells infected with $HIV^{\Delta Nef}$. Therefore, having a high effector:target ratio or longer incubation time may lead to excessive CD8+ T-cell-mediated clearance of the target cells during the viral competition assay. Hence, low effector:target ratios are important to ensure adequate sensitivity in the clearance of the target cells. If excessive CD8+ T-cell-mediated clearance is observed, lower effector:target ratios (e.g., 1:1) or shorter co-culture incubations (e.g., 6 h) can be assessed.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Prof. Sarah Palmer (sarah.palmer@sydney.edu.au).

Materials availability

The study did not generate new unique reagents.

Data and code availability

No data or code was generated or analyzed in this protocol.

ACKNOWLEDGMENTS

This work was supported by the Delaney AIDS Research Enterprise (DARE) to Find a Cure (1U19AI096109, 1UM1AI126611-01, and 1UM1AI164560-01), an amfAR Research Consortium on HIV Eradication (ARCHE) Collaborative Research grant from The Foundation for AIDS Research (amfAR 108074-50-RGRL), and the Australian National Health and Medical Research Council (APP1061681 and APP1149990). G.D. is supported by The Institute for Infectious Diseases, The University of Sydney, Australia. Flow cytometry was performed at the Westmead Scientific Platforms, which are supported by the Westmead Research Hub, the Cancer Institute New South Wales, the National Health and Medical Research Council, and the Ian Potter Foundation. We acknowledge with gratitude the participants who donated samples for this study.

AUTHOR CONTRIBUTIONS

Conceptualization, G.D., S.P.; Investigation, G.D., S.C., S.P.; Writing – Original Draft, G.D., S.C; Writing – Review & Editing, G.D., S.C., S.P.; Resources, A.D.K., S.P.; Funding Acquisition, S.P.; Supervision, G.D., S.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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