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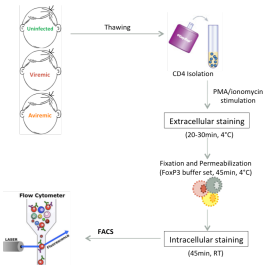
HIV-Flow assay



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Abstract

The purpose of this document is to define the procedure of the HIV-Flow protocol, an assay developed to measure the size the translation-competent viral reservoir in HIV-infected individuals and to phenotypically characterize these cells.

Guidelines

- In each experiment, include PBMCs from an uninfected control donor to set the threshold of positivity
- The frequency of p24 double positive cells (KC57+, 28B7+) is determined by flow cytometry in gated viable T cells. Please note that this gate should include both CD4 positive and CD4 negative T cells (since CD4 is dowregulated by productive HIV infection)

Materials

- 1 bottle (500mL) of RPMI (Cellgro, 10-040-CV)
- 50mL of Fetal Bovine Serum (FBS, PAA, A15-752)
- Penicillin-Streptomycin (Life Technologies, 15070-063)
- 50mL of Bovine Serum (BS)
- PBS 1X pH7.2 (Wisent, 311-013-CL)
- Human Serum (Atlanta Biologicals, 540110)
  Aliquot (5-10mL) and keep at -20°C. After thawing, filter with a falcon cell strainer.
- Trypan Blue 0.4% (Gibco, 15250)
- 50mL PP tubes (BD Falcon, 352098)
- 5, 10 and 25 mL disposable pipettes (BD Flacon, 356542, 357551 and 357525)
- 1.5ml screw-cap microtubes (Sarstedt, 72.692.005)
- Staining buffer: PBS/HS4% (2mL human serum + 48mL of PBS).
- Foxp3 / Transcription Factor Staining Buffer Set (Ebioscience; 00-5523)
- Sterile Water
- EasySep Buffer (StemCell, 20144)
- EasySep Human CD4 T cell enrichment kit (Stemcell, 19052)
- Phorbol 12 myristate 13 acetate (PMA) (Sigma, P8139)
- Ionomycin (Sigma, I9657)
- Brefeldin A (Sigma, B5936-200UL)
- ARV cocktail 100X: Use RPMI to prepare a 100X ARV cocktail solution containing:
  20µM 3TC (for a final concentration of 200nM)
  20µM RAL (for a final concentration of 200nM)
  Aliquot at 200µL in 1.5mL microtubes; Store at -20ºC.
- p24 KC57-PE (Beckman Coulter, 6604667)
- p24 28B7-APC (Medimabs, MM-0289-APC)

Before start

Please note that the p24 antibodies need to be titrated upon reception of each new batch.

Day 1

1 Thaw cryopreserved PBMCs (10-50 ×10^6 cells depending on cell availability and CD4 counts) collected from an HIV-infected individual and an equivalent number of cells from an uninfected control. After thawing, resuspend the cells in 25mL of cRPMI.

2 Count the cells with Trypan Blue.

3 Keep PBMCs from the uninfected control donor for FACS compensations (around 100,000 cells per antibody used).

4 Centrifuge the cells (5min, 1,500rpm). Discard supernatant and break the pellet of cells tapping it.

5 Negative selection of CD4 T cells
According to cell count:

- If more than 50.10^6 cells: Resuspend the pellet with 800µL of Easysep Buffer and complete up to a concentration of 50×10^6 cells/mL.
- If 10×10^6-50×10^6 cells: Resuspend the pellet with 800µL of Easysep Buffer

Transfer the cell suspension in a 14mL round bottom tube.

- Add 25µL/mL of cells of Ab cocktail (19052). Incubate 10min at RT.
- Vortex beads 30s and add 50µL of beads/mL of cells. Incubate 5min at RT.
- Add EasySep buffer up to 5mL (if cell volume <5mL) and up to 8mL (if cell volume >5mL).
- Place the tube (without the cap) in the magnet for 5min.
- Pipet cautiously the cells and transfer them to a 50mL tube containing cRPMI up to 20mL.
- Centrifuge the cells (5min, 1,500rpm), and discard the supernatant

6 Resuspend the cells in 800µL of cRPMI + ARV (Stock ARV = 100X) and count the cells in Trypan Blue.

7 Resuspend the cells at 2×10^6 cells/mL in cRPMI + ARV (Stock ARV = 100X) and incubate at 37ºC 5% CO2 in culture plate
- Less than 200uL: 96-well plate
- 200uL-500uL: 48-well plate
- 500uL-1.5mL: 24-well plate
- 1.5mL-2.5mL: 12-well plate
- 2.5mL-3.5mL: 6-well plate

8 Optional: For immunophenoytping, add BFA (Brefeldin A) to the culture medium (Dilution 2,000X).
- Of note, not all markers will retain their expression after PMA/ionomycin stimulation despite the use of BFA. This should be tested.
- If the goal of the experiment is only to measure the frequency of p24+ (without phenotyping), do not perfom this step.

9 Rest the cells for at least 1hour before proceeding to PMA/Ionomycin stimulation
a) If ART-treated individuals: PMA 162nM, Ionomycin 1µg/mL, 24h
Dilution 10X: 10µL of PMA + 10µL of iono + 80µL of medium
Dilution 100X: 10µL of diluted PMA/iono per mL of cells
b) If untreated individuals: PMA 25nM, ionomycin 1µg/mL, 18h
Dilution 64.8X: 2.3µL PMA + 15µL iono + 132.7µL of medium
Dilution 100X: 10µL of diluted PMA/iono per mL of cells
Incubate cells for 18h or 24h (for samples from untreated or treated individuals, respectively) at 37°C 5% CO2

Day 2

10 Collect the cells in 5mL tubes and wash the wells with PBS. Do not exceed 10×10^6 cells per tube.

11 Centrifuge at 1,800rpm for 3min. Discard the supernatant and resuspend the cells in 200µL of PBS + Vivid (5µL Vivid 1/10 + 195µL PBS/condition).Comment: Resuspend 2µl Vivid with 18µl of PBS = 20µL

12 Incubate the cells for 20-30min at 4ºC

13 Wash once with 800µL of PBS/HS4%. Centrifuge (1,800 rpm, 3min). Discard the supernatant

14 Perform the extracellular staining (using your antibodies for cell-surface antigens of interest) in PBS/HS4% (final volume = 100µL/tube) (20-30min, 4°C)

15 Wash with 1.8mL of PBS/HS4%. Centrifuge (1,800rpm, 3min). Discard the supernatant

16 Fix and Perm with 100µl of FoxP3 FixPerm Buffer (Dilution 1:4, 1mL concentrate + 3mL diluent)(45min, 4ºC)

17 Wash with 1.8mL of FoxP3 PermBuffer (Dilution 10X: 1mL PermBuffer + 9mL H2O). Centrifuge (3min, 2,100rpm). Discard the supernatant

18 Perform the intracellular staining in FoxP3 PermBuffer (final volume = 100µL of dilution C/tube) (45min, RT).
Comments
- Dilution A (KC57): 10-fold dilution (1µL+9µL FoxP3 Buffer)
- Dilution B (p24 28B7): 10-fold dilution (1µL+9µL FoxP3 Buffer)
- Dilution C: add 1µL of each dilution per 100µL (1µL DilA + 1µL Dil B + 98µL of FoxP3 PermBuffer)
- Do not vortex the antibodies and the mixes

Please note that the p24 antibodies need to be titrated upon reception of each new batch.

19 Wash with 1.8mL of FoxP3 PermBuffer. Centrifuge (2,100 rpm, 3min). Discard the supernatant

20 Resuspend cells in 100-200µL of PBS (NO HUMAN SERUM!)

21 Analyze on a Flow Cytometer.