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HIV-Flow assay

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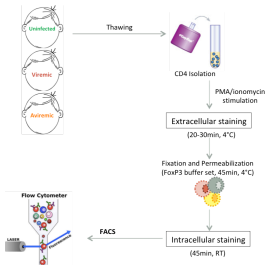

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Abstract

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)

Troubleshooting

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

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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.