1. Principle

Maraviroc (MVC) and Cenicriviroc (CVC) are chemokine receptor antagonists that bind to CCR5 and CCR5/CCR2, respectively. This assay was developed to qualitatively measure the degree to which those chemokine receptors are occupied by drug. When PBMCs from a drug-treated participant are treated with PSC-RANTES and MCP-1, the chemokines bind to the CCR5 and CCR2 receptors that are not occupied by MVC or CVC, and chemokine binding prevents the receptors from labeling by CCR5/CCR2 specific fluorochrome conjugated antibodies. MVC and CVC block chemokine binding to receptor and allow the receptors to be labelled by the fluorochrome conjugated antbodies. By comparing the expression of CCR5/CCR2 antibodies on PBMCs treated in the presence or absence of PSC-RANTES/MCP-1, we can qualitatively measure the degree to which the CCR5 and/or CCR2 receptors are occupied with study drug.

2. Specimen Requirements:

2.1. Primary Specimen: EDTA anti-coagulated peripheral blood mononuclear cells (PBMCs). PBMCs can be fresh or frozen and should be isolated by HANC PBMC Processing SOP and/or thawed by the ACTG/IMPAACT PBMC Thawing SOP.

3. Reagents and Materials:

3.1. RPMI 1640
- 3.1.1. Gibco (Cat # 21870-076) store at 4°C

3.2. Fetal Bovine Serum (Heat Inactivated)
- 3.2.1. Gemini Bio-Products (Cat # 100-500) store at -20°C

3.3. Penicillin/Streptomycin
- 3.3.1. Life Technologies (Cat # 15140-122) store at -20°C

3.4. L-Glutamine (200mM)
- 3.4.1. Life Technologies (Cat # 25030-024) store at -20°C

3.5. Complete Medium (referred to as CM hereafter)
- 3.5.1. RPMI 1640 with 10% HI FBS containing 200 U/mL penicillin and 200 µg/mL streptomycin and 2mM L-glutamine.
- 3.5.2. Store at 4C. Good for one month.

3.6. Dulbecco's phosphate buffered saline (DPBS, e.g. Gibco Cat # 14190-144). Store at room temperature.

3.7. Bleach

3.8. PSC-RANTES (NATIVE RANTES SHOULD ALSO DO THE JOB)
- 3.8.1. 100µM stock received from Oliver Hartley diluted to 1 µM with DPBS. Store at -80C.

3.9. MCP-1 (R&D Systems Cat# 279-MC-10)
- 3.9.1. 10 ug of lyophilized stock diluted with 1mL DPBS. Store at -80C.

3.10. Staining/Wash buffer for antibody staining (flow wash buffer):
- 3.10.1. DPBS containing 0.1% sodium azide (Sigma #S2002) and 1% BSA (Sigma #A7906). Stored at 4°C.

3.11. Fixation Buffer:
- 3.11.1. 1% PFA solution
- 3.11.2. 1:10 dilution of 10% paraformaldehyde (Electron Microscopy Sciences Cat # 15712-S) with DPBS. Stored at 4°C (stable for 6 months).

3.12. Live/Dead Cell Viability Stain
- 3.12.1. Invitrogen Live/Dead Violet/Aqua/Yellow, etc. (panel dependent)

3.13. Conjugated Monoclonal Antibodies:
- 3.13.1. CCR5 2D7 PE (BD Cat# 555993) and isotype (Cat# 555574)
- 3.13.2. CCR2 APC (BioLegend Cat# 357208) and isotype (Cat # 400222)
- 3.13.3. CD3/CD4/CD8/CD14 and any additional markers will be defined by the protocol

3.14. Study Drug
- 3.14.1. Only needed if doing a positive control
- 3.14.2. CVC or MVC were diluted to 10uM by the following series of dilutions:

- Add enough DMSO to the powdered drug to get to a concentration of 10mM (This will depend on weight of sample and molecular weight of drug. This website could be helpful: https://www.graphpad.com/quickcalcs/Molarityform.cfm)
- 25µL of 10mM stock + 50µL of DMSO + 25µL PBS = 2.5mM
- 20µL of 2.5mM stock + 480µL of 75% DMSO in PBS = 100 µM
- 20µL of 100µM stock + 180µL of CM = 10µM
- Solution stored at 4°C for up to 7 days

4. Equipment

4.1. Class 2 Biosafety Cabinet
4.2. Centrifuge
4.3. Refrigerator 4°C
4.4. Freezer -80°C
4.5. Falcon 12mm x 75mm tubes with snap cap (Cat#352063)
4.6. 24-well plate (optional)
4.7. Micropipettes capable of dispensing 20µL, 100µL, 1000µL
4.8. Disposable pipette tips
4.9. Interval timers
4.10. Disposable Gloves
4.11. Lab coat
4.12. Safety Glasses
4.13. Flow cytometer capable of at least 7-color detection

5. Instrumentation

5.1. Each laboratory will have a comprehensive quality assurance program that will assure proper instrument operation, calibration stability and personnel training.

6. Procedure

6.1. The number of conditions needed will depend on the study design
  6.1.1. One tube will be needed as a control, one will be needed for PSC-RANTES, and another will be needed for MCP-1 (if necessary)
  6.1.2. The number of tubes will need to be doubled if incubating with study drug as a positive control
  6.1.3. 1-2 x 10^6 PBMCs will be needed per tube/condition
6.2. Positive control (if desired) incubated with drug
  6.2.1. Wash PBMCs (fresh or thawed) once with CM and spin at 400xg
  6.2.2. Resuspend 1-2x10^6 PBMCs in a 5mL flow tube with snap cap (or a 24-well plate) in 990µL of CM
  6.2.3. Add 10µL of 10µM study drug to three positive control tubes for a final concentration of 100nM
  6.2.4. Incubate all tubes for one hour at 37°C
  6.2.5. Add 1mL of CM and spin for 10 minutes at 400xg
  6.2.6. Wash with 2mL of CM and spin for 10 minutes at 400xg
6.3. Incubation with PSC-RANTES and/or MCP-1
  6.3.1. Add 990µL of CM to each tube
  6.3.2. Add 10uL of CM to control tubes
  6.3.3. Add 10uL of 1 µM PSC-RANTES to the PSC-RANTES tube/s for a final concentration of 10nM
  6.3.4. Add 10uL of 100ug/mL MCP-1 to the MCP-1 tube/s for a final concentration of 100ng/mL
  6.3.5. Incubate for one hour at 37°C
  6.3.6. Wash by adding 1 mL of PBS
  6.3.7. Wash with 2 mL of PBS and proceed to Live/Dead staining, followed by surface antibody staining as per your usual flow staining protocol