Isolation of PBMC's from whole blood:

Histopaque procedure: <u>This procedure should be done in the cell culture hood to maintain sterility.</u>

1. To a 15-ml conical centrifuge tube, add 6.0 ml HISTOPAQUE®-1077 and bring to room temperature.

2. Carefully layer 6.0 ml whole blood onto the HISTOPAQUE®-1077. Centrifuge at 400 x g for exactly 30 minutes at room temperature. Centrifugation at lower temperatures, such as 4°C, may result in cell
clumping and poor recovery.

3. After centrifugation, carefully aspirate, with a Pasteur pipet, the upper layer to within 0.5 cm of the opaque

3. Remove 50 ul of cells (with or without Fc Block) and incubate in a 1.5 ml eppendorf tube. Add appropriate amount of antibody into almost all tubes (one no antibody control needed, other controls may be needed). Incubate for at least 30 minutes in the dark on ice or at 4°C.

4. Add 200 ul of Flow Cytometry Staining Buffer to each tube tostop the reaction. Pellet the cells by centrifugation at 5000 rpm for 5 minutes. Decant the supernatant. Resuspend the pellet in 100 ul of staining buffer. Pulse vortex to resuspend the pellet. If also staining intracellular antigens proceed to step 6.

5. Add 5 ul of Propidium iodide (1 mg/ml solution) to identify dead cells. Run on flow cytometer.

Intracellular Staining

1. Fix the cells by adding 100 µL of IC Fixation Buffer (eBioscience) while vortexing the tube.

2. Incubate in the dark at room temperature for 20 minutes.

3. Without washing, add 1 mL of 1X Permeabilization Buffer (eBioscience) to each tube.

4. Centrifuge samples at 5000 rpm at room temperature for 5 minutes, then discard the supernatant.

5. Resuspend the cell pellet in 1 mL of 1X Permeabilization Buffer. RESUPSEND THE PELLET!

6. Centrifuge samples at 5000 rpm at room temperature for 5 minutes, then discard the supernatant.

7. Resuspend the cells in 100µL of 1X Permeabilization Buffer to each tube. Add appropriate amount of antibody per sample and incubate in the dark at room temperature for 20 minutes.

8. Add 1 mL of 1X Permeabilization Buffer to each tube.

9. Centrifuge samples at 5000 rpm at room temperature for 5minutes, then discard the supernatant.

10. Add 1 mL of Flow Cytometry Staining Buffer to each tube.

11. Run on flow cytometer. 30,000 events per sample