Indirect flow cytometry protocol for human primary isolates PBMCs and Whole Blood

1. Harvest and wash the cells then determine the total cell number.

   Cells are usually stained in polystyrene round bottom 12 x 75 mm^2 Falcon tubes. However, they can be stained in any container for which you have an appropriate centrifuge e.g. test tubes, eppendorf tubes, and 96 well, round-bottomed microtiter plates. In general, cells should be spun down hard enough so that the supernatant fluid can be removed with little loss of cells, but not so hard that the cells are difficult to resuspend.

2. **If you are using whole blood, AMREPFlow strongly recommend using a Red Blood Cell (RBC) Lysis Buffer (either hypotonic, ammonium chloride or any commercial lysis buffers will suffice). RBCs can and will overshadow (or swamp) your population of Leukocytes, making them hard to clearly indentify**.

   It is always useful to check the viability of the cells which should be around 95% and not less than 90%.

3. Resuspend the cells to approximately 1 x 10^6 cells/ml in ice cold PBS, 10% FCS, 1% sodium azide.

Use ice cold reagents/solutions and keep cells at 4°C as low temperature and presence of sodium azide prevent the modulation and internalization of surface antigens which can produce a loss of fluorescence intensity.

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4. Add 100 µl of cell suspension to each tube.
5. Add 0.1-10 µg/ml of the primary antibody. Dilutions, if necessary, should be made in 3% BSA/PBS. The amount of antibody that needs to be added should be determined by an antibody titration step. (https://www.amrepflow.org.au/education/antibody-titration)
6. Incubate for at least 30 min at room temperature or 4°C in the dark.
7. Wash the cells 3 times by centrifugation at 400 g for 5 min and resuspend them in ice cold PBS. You may need to adjust the conditions of the centrifugation (force and time) for the cell types used.
8. Dilute the fluorochrome-labeled secondary antibody in 3% BSA/PBS at the optimal dilution (according to the manufacturer's instructions) and then resuspend the cells in this solution. Always include a secondary only antibody control to your control panel, to ascertain if there is any non specific binding.

9. Incubate for at least 20-30 min at room temperature of 4°C. This incubation must be done in the dark.
10. Wash the cells 3 times by centrifugation at 400 g for 5 min and resuspend them in 500 µL to 1 mL of fixative (such as 0.5-1% paraformaldehyde for 15-30mins. For known infectious samples, use 3-4% paraformaldehyde for 2-3 hours).
    10.1. Always wash out the fixative post usage as this can affect fluorophore stability and fluorescence intensity.
    10.2. **For general immunophenotyping purposes if you're using AMREPFlow instruments you MUST fix your sample.**
    10.3. **If you require to perform functional studies on live human samples, you must provide a Risk Assessment Document which will be reviewed by the Flow OHS Committee before work can commence.**