Cell preparation and staining procedure

A. Whole blood lyse

Whole bloods lysing techniques are useful because they are rapid, require minimal handling of blood and can be performed using relatively small volumes of blood. This is complemented by the lower potential for differential loss of specific lymphocyte sub populations when compared to density gradient separation techniques. The lysing agent is intended to lyse red cells, yet leaving the leukocyte population intact. It is imperative that the lysing agent does not alter the surface antigens of the white cells and the debris from the red cell hemolysis does not interfere with the white cell analysis.

Several limitations of the method that should be noted:

1. Abnormal red blood cells (e.g., nucleated red blood cells, and cells from patient that have had a splenectomy) may be resistant to lysing.
2. Granulocytes may compete with lymphocytes when using certain monoclonal antibodies that may result in sub-optimal staining.
3. The presence of plasma in the blood samples may interfere with the binding of some antibodies (e.g., soluble substances binding to the monoclonal antibodies or blocking cell surface receptors, reaction of human anti-murine antibody (HAMA) with the monoclonal and serum immunoglobulins).
4. Since the cell count is not standardized prior to staining, the amount of antibody relative to the cell number may not be optimal.
5. This method assumes that all lymphocyte subsets are equally resistant to the lysing method.

Each day a normal adult volunteer must be drawn, stained and run in conjunction with the patient samples.

NOTE: Observe Universal Precautions handling all specimens.

A.1 Preparing the blood

1. Invert the EDTA tube with 2 ml of blood several times to mix the specimen; 100 µl of blood is required for each staining tube. Place the 2 ml of whole blood in a 15 ml conical tube labeled with the patient's name. Fill the tube to 15 ml with PBS. Cap the tube and invert several times to mix.

2. Place the tube of diluted blood in the centrifuge and spin for 10 minutes at 1000 RPM at 18-22^{O} Room temperature (RT)

3. Aspirate the fluid from the tube down until you have around 2 ml left.

A.2 Directly labeled monoclonal staining procedure

Panel currently used by our lab (NIH Immunology Service) for initial screening:

Tube	FITC	PE	PerCP	PE-Cy7	APC	APC-efluor 750
1	CD14					CD45 Ungated
2	CD14					CD45
3	IgG	IgG	IgG	IgG	IgG	CD45
4	HLA DR	CD25	CD4	CD3	CD8	CD45
5	CD4+CD8	Alpha/Beta	CD3			CD45
6	CD4+CD8	Gamma/Delta	CD3			CD45
7	CD3/CD16+CD56					CD45
8	CD19	CD5	CD20	CD3	CD27	CD45

1. Label 12 X 75 mm polystyrene tubes

2. Prewet the tubes with 0.5-1.0 ml of 5% FCS in PBS. Decant fluid and wick the tubes dry.

3. Add antibodies to the bottom of tubes using a fresh pipette tip for each antibody. (The volume of the antibodies required should be noted in the product insert sheet).

4. Add 100 µl of well mixed washed whole blood to the bottom of each tube containing the monoclonal antibodies. Remove any extraneous blood from the side of the test tube by using a cotton tipped applicator.

5. Mix the cells and the antibodies together by gently drawing the tubes across the back of the white plastic test tube rack or gently vortex.

6. Incubate in the refrigerator (in the dark) at $4^{o}C$ for 30 minutes. The cold temperature and azide prevent shedding, capping or internalization of the monoclonal antibodies.

7. Add 2 ml of BD Lysing solution to each tube using the dispensette attached to the working stock (1:10 dilution) lyse reagent.

8. Vortex thoroughly. Place tubes in the dark at RT for 10 minutes. The timing is very critical as over-lysing may damage the white cells and under-lysing will result in red cell contamination.

9. Place the tubes in the centrifuge and spin for 5 minutes at RT and 1000 RPM.

10. Invert the tubes and carefully decant the supernatant, then wick dry by placing the tubes on plastic backed gauze. (Decanting and wicking must be done with a single inversion to minimize disturbing the cell button.)

11. Vortex to re-suspend the cells

12. Add 2 ml of PBS to each tube. Centrifuge again for 5 minutes at 1000 RPM.

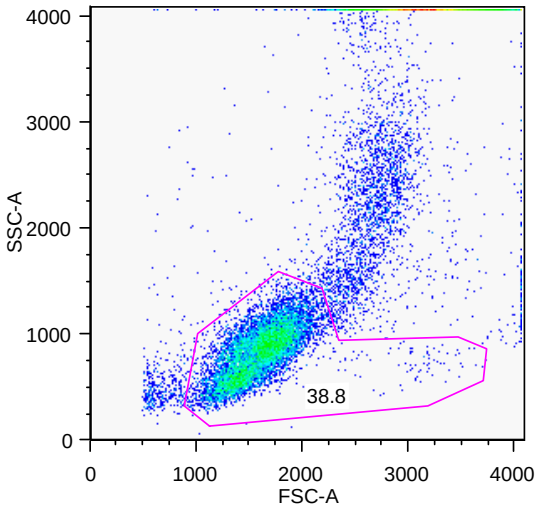
13. Decant fluid from the tubes and wick dry. Vortex to resuspend the cells.

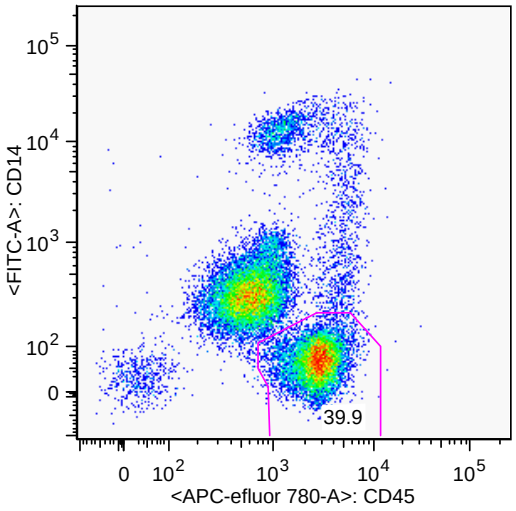
14. Fix cells by adding 100 to 200 µl of 1.0% formaldehyde.

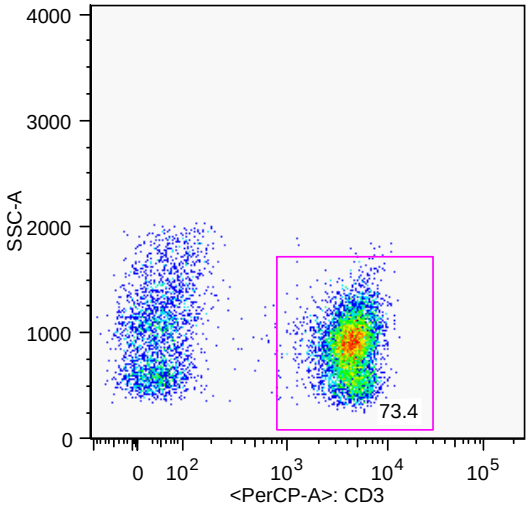
The cells in suspension are now ready to be acquired and analyzed on the flow cytometer. The tubes may be stored at $4^{o}C$ covered with aluminum foil labeled with the date they were prepared and the patient's name. Optimally the cells should be analyzed within 24 hours. They can be saved for up to 72 hours, but there may be diminished fluorescence and/or cell degradation.

A.3 Typical results

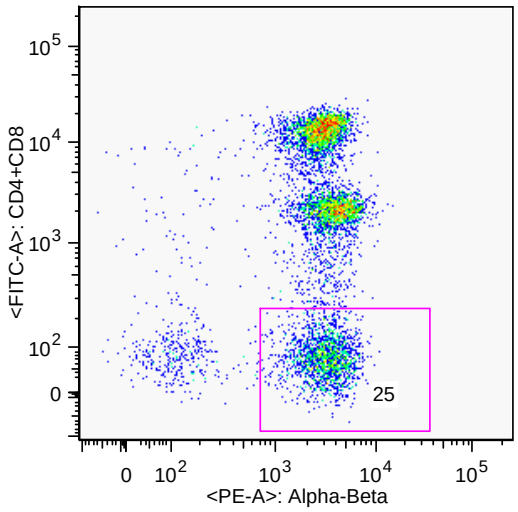
Lymphocyte gating can be performed by FSC x SCC or CD14 x CD45. We currently include CD4 in every tube, to guarantee a high level of purity and accurate numbers. For DNT evaluation, one can subsequently gate on CD3+ cells and plot TCR$\alpha\beta$ x CD4+CD8 (same color) cells, as demonstrated below.







ALPS



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