PROCEDURAL SUGGESTIONS FOR SURFACE ANTIGEN STAINING OF CELLS FOR FLUORESCENCE ACTIVATED CELL SORTING

I. SAMPLE (one or more of the following preparations)

A. Suspensions of single cells from tissues

B. Tissue culture cells, including adherent cells detached from support

C. Ficoll-hypaque separated mononuclear cells

II. MATERIALS

A. Antibodies

1. Primary antibodies: directly labeled with a fluorochrome.

2. Secondary antibodies: fluorochrome-labeled Streptavidin or antisera.

B. 1 X PBS + 2% newborn calf serum + 0.1% sodium azide (buffer)

C. Refrigerated centrifuge.

OPTIONAL:

A. Ammonium chloride lysing solution (see attached preparation method and procedure)

B. RPMI 1640 media containing 10% or 20% serum

Staining procedure

1. Place up to 30 X 10^6 Ficoll-hypaque separated cells in a 12 X 75 mm polypropylene sample tube and wash them once with buffer.

2. Add 0.5 mL of buffer to the pellet; then add the appropriate amounts of the monoclonal antibodies, e.g., FITC, PE, biotinylated or unconjugated antibodies. Usually 1/5 to 1/4 of the amount recommended by the manufacturer per cell number is sufficient for staining.

3. Vortex briefly and incubate for 30 min at 4°C or for 15 min at room temperature in the dark.

4. Wash once with 3.5 mL of buffer by centrifugation at 250g for 10 min. Aspirate supernatant. Resuspend the pellet in 3 mL of buffer.

5. If you need to add a second step reagent, add 150 µL of buffer to the pellet, then add the appropriate amount of labeled reagent (for avidin, usually 1/4 of the recommended amount is sufficient). Vortex briefly and incubate for 20 to 30 min at 4°C.

6. Wash once with 3.5 mL of buffer by centrifugation at 250g for 10 min, then aspirate supernatant and resuspend in 3 mL of buffer.

Note: if you have more than 30 X 10^6 cells to stain, use separate tubes for staining of 30 X 10^6 each. Also, do not start to stain all cells at the same time, wait with the second tube until the first one is done. Thus, the intensity of staining will not fade until all your cells are sorted. For staining of less cells, scale down the reagent and the buffer, e.g., to stain 10 X 10^6 cells use ~170 µL of buffer and the appropriately scaled-down amount of antibodies.

Re-suspend cells at a concentration of 10-20 X 10^6 for the actual sort. For cell preparations that tend to clump excessively, a cell concentration of approximately 5 X 10^6 can reduce clumping. If your cell number is low, resuspend the sample in at least 0.4 mL of buffer. Buffering with HEPES at a conc of 25mM is preferable over using a bicarbonate buffer, and the serum conc should not exceed 2-3%, because high serum interferes with the formation of tight sort streams

It is possible to use RPMI 1640 that contains serum as staining buffer. Sodium azide is not needed as long as the incubation and the washes are done at 4°C. However, we have found that RPMI can increase auto-fluorescence of cells and is not necessary for good viability (at least for human lymphocytes). Do not use RPMI for staining with avidin, because the biotin it contains will bind all the avidin.

If your cell preparation visually contains residual red blood cells, you have to lyse these red cells with ammonium chloride according to the attached protocol. Excessive red cells can interfere with the purity of your sort and can appear in the sorted population.

For a sterile sort, it is necessary that staining be done under sterile conditions. Unopened bottles of antibodies do not need to be filter-sterilized. If you have to filter your reagents, use 0.22 micron Millipore disk filters that fit on small syringes and minimize your reagent loss. However, you will still lose some of your reagent.

LYSING PROCEDURE FOR LYSIS OF RESIDUAL RED BLOOD CELLS

IN FICOLL-HYPAQUE SEPARATED MONONUCLEAR CELLS

1. After the staining procedure, take off as much of the washing buffer as possible without disturbing the pellet.
2. Vortex briefly and add 1 mL of room temperature 1X NH_4Cl lysing solution to your cells, vortex again and expose your cells to it for 2-3 minutes at room temperature. Note: do not exceed this time.
3. Centrifuge for 5 minutes at 200g. Remove the lysing buffer completely and resuspend in BUFFER or 1% formaldehyde solution for analysis.

AMMONIUM CHLORIDE LYSING SOLUTION – 10X

Reagents	Amount
Ammonium Chloride, ACS	82.9 g
Potassium Bicarbonate, USP	10.0 g
Ethylenediamine tetraacetic acid (EDTA) disodium salt	0.37 g
Water, glass distilled	qsad 1.0 liter

Adjust pH to 7.2 and keep in tightly closed container at 4°C. To prepare a 1X working solution (to be used at room temperature), dilute 10X 1:10 with glass distilled water. Keep tightly closed and discard at the end of the day.