MONOCLONAL ANTIBODY STAINING PROCEDURE

I. SAMPLE – one or more of the following preparations

A. Suspension of single cells from tissue (e.g., lymph node, spleen, bone marrow, placental cells)

B. Tissue culture cells (including adherent cells detached from support)

C. Ficoll – hypaque separated mononuclear cells

II. REAGENTS

A. Antibodies

1. Primary antibodies: purchased or your own

a. if these are conjugated to a fluorochrome (e.g., FITC, PE, or other) use the DIRECT STAINING PROCEDURE

b. if the primary antibody is not conjugated to a fluorochrome, use the INDIRECT STAINING PROCEDURE

2. Secondary antibodies: fluorochrome-conjugated polyclonal antibodies

B. BUFFER: Phosphate-buffered saline (PBS Ca^{2+} and Mg^{2+} free) + 2 % newborn calf serum (or 0.2% BSA) + 0.1% sodium azide.

C. Formaldehyde preservative: 2% solution in protein-free PBS

D. PBS: protein and azide free

III. TUBES

A. Use 12 x 75 mm polystyrene/polypropylene tubes. Please note that Falcon snap-cap tubes are the only ones that for certain fit on the BD Bioscience flow cytometers, although others may be also suitable. If you want to stain in another tube type or in plates, please transfer the cells finally to those required.

B. Mark all tubes with easily readable numbers or designations corresponding to your protocol sheet.

C. Use 1% formaldehyde solution for re-suspension of all potentially biohazardous specimens and cap them.

IV. PROTOCOL

DIRECT STAINING PROCEDURE

1. Prepare your cells as a suspension of single cells in a manner appropriate for the specimen you wish to examine. Make sure the cells are viable. As the final step, wash at least once with 1 mL of cold BUFFER. Resuspend the cells at 10^7 cells/ml (thus 50 µL = 5 x 10^5 cells) in cold BUFFER.

2. Meanwhile add 50 µL of BUFFER and then the appropriate amount of monoclonal antibody to the bottom of tubes. Note: For multi-color staining, add all your fluorochrome-conjugated antibodies at the same time.

3. Add 50 µL of the cell suspension to the bottom of the tubes.

4. Vortex briefly and incubate 30 minutes at 4° C in the dark.

5. Wash twice with 1 ml of buffer; centrifuge at 250g for 5 minutes.

6. Resuspend samples in 0.5 mL of buffer and hold at 4°C in the refrigerator (or on ice) prior to analysis.

INDIRECT STAINING PROCEDURE

1-5. Process cell samples as above using a working dilution of unlabelled monoclonal antibody.

6. Resuspend cell pellet in 100 µL of working dilution of the fluorochrome-conjugated secondary antibody.

7. Vortex briefly and incubate for 20 minutes in the refrigerator.

8. Wash twice with ~ 1 mL of buffer; centrifuge at 250g for 5 minutes.

9. Resuspend samples in 1 mL of buffer and hold at 4°C in refrigerator (or on ice) protected from light prior to analysis.

NOTE: If your samples are Ficoll-Hypaque-separated PBMC you might still have residual red blood cells in your preparation. Red cells interfere with flow cytometric analysis of lymphocytes and have to be removed by NH4Cl lysis. See recipe and procedure below.

PRESERVING PROCEDURE

If cells are not going to be read on the flow cytometer on the same day, or they are considered potentially biohazardous, do not re-suspend them after the staining procedure. Stop at Step 5 of DIRECT STAINING or at Step 8 of INDIRECT STAINING, and continue as below.

1. Add to the pellet 0.25 mL of cold protein-free PBS, and vortex; then add 0.25 mL of cold formaldehyde solution (see attached recipes).

2. Vortex again and incubate in the refrigerator. Make sure to keep cells in the dark as exposure to light may cause loss of fluorescence.

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. **Note** that 10X NH4CL Lysing solution can be obtained commercially, e.g. from BD Pharmingen, CA, PharM Lyse™ Cat# 555899.

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.

PREPARATION OF 2% FORMALDEHYDE STOCK SOLUTION (2 METHODS)

METHOD 1:

Formaldehyde preservative – 2% formaldehyde solution in protein-free PBS.

Prepare as follows:

10% formaldehyde*	20 ml
10 x PBS	10 ml
Distilled water	70 ml

* 10% formaldehyde solution (e.g., Polysciences Inc., Warrington, PA, Ultrapure, Cat.#04018), depolymerized paraformaldehyde, EM grade, methanol-free solution.

METHOD 2:

Formaldehyde preservative – 2% formaldehyde solution in protein-free phosphate-buffered saline (PBS).

Prepare as follows:

Add 2 g paraformaldehyde powder (e.g., Sigma, St. Louis, MO) to 100 mL of 1 X PBS. Heat to 70°C (do not exceed this temperature) in a fume hood until the paraformaldehyde goes into solution (note that this happens quickly as soon as the suspension reaches 70°C). Allow the solution to cool to room temperature. Adjust to pH 7.4 using 0.1 M NaOH or 0.1 M HCl, if needed. Filter and store at 4°C protected from light.