I. GENERAL ANTIBODY STAINING TECHNIQUE.

A. PRINCIPLE.

There are two general types of antibody staining: The first is an ***indirect technique*** in which cells are incubated with an unconjugated primary antibody. The primary antibody recognizes and binds to cell surface antigens but because there is no way to detect it's presence on the cell surface, the cells are subsequently incubated with a fluorochrome conjugated secondary antibody. The secondary antibody recognizes and binds to only the primary antibody molecule to provide a fluorescent reporter for the primary antibodies bound to the cell surface. The two step indirect stain offers an amplification of signal in that several secondary antibodies can bind to a single primary antibody and is therefore generally used for single color immunofluorescence of low density antigens.

The second type of stain is a ***direct technique*** in which cells are incubated with fluorochrome conjugated antibody/ies which recognize and bind to various cell surface antigens. The direct stain offers the advantage of reduced steps in the staining procedure and therefore a savings of time. The antibodies are almost always monoclonal antibodies (MoAb) and they may be added either singly for single color fluorescence, or in multiple combinations for multi-color fluorescence. It is possible to combine an indirect stain with a direct stain for multi-color fluorescence (see section "E. Limitations of Procedure").

After staining, the cell sample is washed and analyzed in the flow cytometer to enumerate cells expressing the antigen in question and make observations on the quantity of antigen per cell (intensity of fluorescence).

B. SPECIMEN.

1) Peripheral blood, bone marrow or buffy coat cells. Ideally, the specimen should be processed within 6 hours of collection for best results. If longer storage is necessary, samples should be kept refrigerated (4-6 C°) and should be processed within 24 hr of collection for optimal results.

2) Cell suspensions. Tissue isolates or cultured cells may be kept in the tissue culture medium or balanced salt solution in which they were prepared but should be refrigerated (4-6 C°) until staining.

C. REAGENTS.

Antibodies (polyclonal or monoclonal) pre-titered for optimal staining
12 x 75 mm Falcon polystyrene tubes
PBS-BSA = PBS (Ca^{++}/Mg^{++} free) containing 0.5% BSA, pH 7.2-7.4
PBS-FIX = PBS containing 1% paraformaldehyde, pH 7.2-7.4
Lysing reagent (commercial or locally prepared NH_4Cl)

D. PROCEDURE.

1) Label tubes with the antibody/ies that are to be added to that tube.

2) Add the appropriate volume of monoclonal antibody/ies to each tube. Alternatively, the antibody/ies may be pre-mixed in PBS-BSA to create a "working dilution" so that 100µl can be dispensed to each tube containing the correct volume of antibody/ies. The latter approach is more efficient when staining several tubes with multi-color combinations.

3) Add 100µl of a 1 x 10^7 /ml suspension of cells to each tube and mix gently by vortexing. If staining whole blood, bone marrow or buffy coat cells add a volume of that product

which will contain about 1-2 x 10^6 of the cells of interest.

4) Incubate in the dark for 20 min with occasional mixing. Incubation may be at room temperature (20-25 C°) for whole blood or at 4°C for purified lymphoid cell suspensions. ***For direct stains skip to step 8.***

5) Wash 2X with 2 ml PBS-BSA at 400 x G for 3 min.

6) To each cell pellet add the appropriate amount of secondary antibody (ex. goat -mouse FITC) in 100µl of PBS-BSA, and mix gently by vortexing.

7) Incubate in the dark for 30 min at room temperature (20-25 C°) for blood-buffy coat or at 4°C for purified lymphoid cell suspensions.

8) For whole blood, bone marrow or buffy coat cells; lyse the red cells by adding 3 ml of warmed NH_4Cl and mix by vortexing at least 10 seconds. Incubate 10 min at room temperature (20-25 C□), centrifuge at 400 x G for 3 min and decant the supernatant. For purified cell suspensions; skip this step and proceed on to next step.

9) Wash 1X with 2 ml PBS-BSA at 400 x G for 3 min.

10) Resuspend in 0.5 ml of PBS-BSA (or PBS-FIX if samples will not be analyzed until the next day). All specimens should be stored at 4°C and protected from light until analyzed.

11) If cell aggregates are present, filter the suspension through 40µ nylon screen just prior to analysis.

E. LIMITATIONS OF PROCEDURE.

1) Indirect staining cannot be used for dual staining with two primary murine MoAbs as is commonly done with direct stains. The secondary antibody (GAM-FITC) would not distinguish between the two primary MoAb's and would bind to both.

2) A single indirect stain can be used in conjunction with a direct stain if the complete indirect staining process (through GAM-FITC) is done first followed by a wash step containing 10µl of mouse IgG to block remaining binding sites on the GAM-FITC. The directly conjugated MoAb is then added to the suspension, incubated as described from step 2 of the protocol.

3) Whole blood, bone marrow or buffy coat samples which have been stored in the cold may not lyse well.

F. REFERENCES.

1) *Monoclonal Antibody Source Book*; Section 2, Becton Dickinson Procedures.

2) *Cell Surface and Intracellular Antigens*, pp. 289-297 in "Practical Flow Cytometry" by Howard M. Shapiro, 3rd Ed, Alan R. Liss, 1995.

3) *Phenotypic Analysis*, Section 6.2 Immunophenotyping, in *"Current Protocols in Cytometry"* edited by Robinson, J.P. et al., John Wiley & Sons, NY, 1998.