


# **CLINICAL IMMUNOPHENOTYPING BY FLOW CYTOMETRY**

*Carleton C. Stewart, PhD and Sigrid J. Stewart*

**Laboratory of Flow Cytometry,  
 Roswell Park Cancer Institute, Buffalo, NY**

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## **Introduction**

Immunophenotyping is the term applied to the identification of cells using antibodies to

antigens expressed by these cells. These antigens are actually functional membrane proteins involved in cell communication, adhesion or metabolism. Antibodies bind to three dimensional molecular structures on these antigens called epitopes and each antigen contains several hundred different epitopes. A monoclonal antibody is specific for a single epitope, while polyclonal antibodies, are actually the natural pool of several hundred monoclonal antibodies produced within the animal, each one binding to its unique epitope. Immunophenotyping using flow cytometry has become the method of choice in identifying and sorting cells within complex populations. Applications of this technology have occurred in both basic research and clinical laboratories. The National Committee for Clinical Laboratory Standards has prepared guidelines for Flow Cytometry that describe in detail the current recommendations for processing clinical samples (H42 Landay, 1992 and H43, Borowitz, 1993).

<sup>1</sup>Portions of this text are taken verbatim from: Methods in Cell Biology, Volume 41, Flow Cytometry, Second Edition Part A, edited by Zbigniew Darzynkiewicz, J. Paul Robinson, and Harry A. Crissman, pp. 40 -78, 1994 and from Methods in Molecular Biology, Volume 45, Monoclonal Antibody Protocols, edited by William C. Davis. pp. 129 - 147, Humana Press, 1995. These references may be consulted for more information not presented here.

## Methods

There are three basic methods for staining cells. These three methods can be combined to stain cells with multiple antibodies using different colored fluorochromes. We recommend receiving all samples at room temperature and processing them on ice in dim ambient light until fixed.

### Basic Method 1: Primary antibody followed by fluorochrome conjugated second antibody.

1. Add 50  $\mu$ l of cells ( $5-20 \times 10^6$ /ml) to a 12 x 75 mm tube.
2. Add 10  $\mu$ l IgG fraction (2mg/ml) derived from the same species as the second antibody to the cells and incubate 10 min. This IgG will block Fc receptors and non-specific binding.
3. Add appropriate amount of primary antibody to cells and incubate 15 min.
4. Add 3.5 ml PBS, centrifuge cells at 1500 x g for 15 min., decant supernatant, blot excess buffer on an absorbent towel and resuspend cells in residual buffer. This process will subsequently be called a **wash**.
5. If erythrocyte lysis is desired, substitute the lysis reagent for PBS in step 4.
6. Add the appropriate amount of fluorochrome conjugated second antibody, incubate 15 min. and wash.
7. Resuspend cells in 0.5 ml of 2% ultrapure formaldehyde. [If it is desired to analyze viable cells, substitute the desired resuspension medium].

### Basic Method 2: Biotinylated primary

### antibody followed by fluorochrome conjugated Avidin.

1. Add 50  $\mu$ l of cells ( $5-20 \times 10^6$ /ml) to a 12 x 75 mm tube.
2. Add 10  $\mu$ l IgG fraction (2mg/ml) derived from the same species as the primary biotinylated antibody to the cells and incubate 10 min. This IgG will block Fc receptors and non-specific binding.
3. Add appropriate amount of biotinylated primary antibody to cells and incubate 15 min. and wash. If erythrocyte lysis is desired, substitute the lysis reagent for PBS.
4. Add the appropriate amount of fluorochrome conjugated streptavidin, incubate 15 min. and wash.
5. Resuspend cells in 0.5 ml 2% ultrapure formaldehyde. [If it is desired to analyze viable cells, substitute the desired resuspension medium].

### Basic Method 3: Directly Conjugated Primary Antibody

1. Add 50  $\mu$ l of cells ( $5-20 \times 10^6$ /ml) to a 12 x 75 mm tube.
2. Add 10  $\mu$ l IgG fraction (2 mg/ml) derived from the same species as the conjugated antibody to the cells and incubate 10 min. This IgG will block Fc receptors and non-specific binding.
3. Add appropriate amount of conjugated antibody to cells and incubate 15 min. and wash. If erythrocyte lysis is desired, substitute the lysis reagent for PBS then wash again with PBS.
4. Resuspend cells in 0.5 ml 2% ultrapure

formaldehyde. [If it is desired to analyze viable cells, substitute the desired resuspension medium].

**Multicolor Immunophenotyping For Up To Four Antibodies Simultaneously using Basic Method 3.**

1. Add 50 µl of cells (5-20 x 10<sup>6</sup>/ml) to a 12 x 75 mm tube.
2. Add 10 µl IgG fraction (2 mg/ml) derived from the same species as the antibody to the cells and incubate 10 min. This IgG will block Fc receptors and non-specific binding.
3. Add appropriate amount of each directly conjugated, properly titered antibody to cells and incubate 15 min. and wash. If erythrocyte lysis is desired, substitute the lysis reagent for PBS then wash again with PBS.
4. Resuspend cells in 0.5 ml 2% ultrapure formaldehyde. [If it is desired to analyze viable cells, substitute the desired resuspension medium].

**Precautions:**

Antibodies interact with cells in three ways: first by binding specifically to their epitope, secondly by binding to Fc receptors and thirdly by binding non-specifically. Often Fc receptor binding and nonspecific binding are referred to as nonspecific binding to distinguish between specific binding of the F(ab) portion of the antibody to its epitope. Actually Fc receptor binding of IgG's to cell Fc receptors is very specific and it, like epitope binding, is saturable. In contrast, nonspecific binding exhibits lower affinity binding and it is not saturable.

It is possible to evaluate the extent of the inappropriate binding component using an isotype control. Each antibody should be titered prior to use so that the correct amount to optimally stain the cells is used. Our general practice is to have the optimal amount in 10 µl. Commercially available antibodies that have already been titered may be diluted differently by the supplier so that more or less than 10 µl is required. In these cases, use the suppliers recommended amount (unless it is found to be incorrect by titering). This control consists of the same cells, incubated with a myeloma protein having no epitope specificity, but having the same isotype and subclass as the primary antibody. This myeloma protein is presumed to bind to cells in the same way immunoglobulins bind excluding epitope binding. The percentage of positive cells revealed by the isotype control is then used to set markers for analysis (described in detail below).

The autofluorescence control should also be analyzed. It contains no antibodies and is otherwise processed the same way as the other samples. This control provides a baseline to determine the minimum fluorescence. Ideally, the isotype control and autofluorescence control would give identical results and to the extent they differ is indicative of a problem.

All second antibodies should be F(ab')<sub>2</sub> fragments to eliminate Fc binding. These reagents are currently available against virtually all mammalian species and conjugated with all the common fluorochromes. A whole blood (or bone marrow) lysing method should be used to remove erythrocytes and there are several reagents commercially available. While all of them work with blood, some are better than others for lysing bone marrow erythroid cells. Cells obtained from other sources such as lymph nodes or plural fluid generally do not need to be lysed.

Lysing reagent:

4.13 g Ammonium Chloride (NH<sub>4</sub>Cl) --  
Sigma A-5666

0.5 g Potassium Bicarbonate (KHCO<sub>3</sub>) -  
- Sigma P-4913

0.0185 g Tetra Sodium EDTA -- Sigma  
ED4SS

Measure carefully the above chemicals. Pour into a flask (must be at least a 500 ml flask) to which a stir bar has been added. Add 500 ml double distilled water. Place on stir plate and stir until the powder is dissolved. Do not pH. Use at room temperature.

This reagent must be prepared daily because the dissolved CO<sub>2</sub> in water combines with NH<sub>4</sub>Cl to form the carbonate (NH<sub>4</sub>)<sup>2</sup> CO<sub>3</sub> that is ineffective in lysing erythrocytes. We recommend weighing the reagents and storing them as packets. The dry reagents are dissolved in water as required. Quantities for daily use can be made by scaling the listed reagents up or down.

Paraformaldehyde when dissolved in aqueous solution becomes equivalent to ultrapure formaldehyde. This product is available as a 10% solution from Polysciences Inc. Do not use any other kind of formaldehyde for fixing cells because their autofluorescence can markedly increase.

Any of the three basic methods can be combined to stain cells with antibodies using different colored fluorochromes. There are two rules that must be followed to prevent artefactual data. Method 1, if used, **must always be performed first**. The second antibody must be blocked by IgG from the **same species as the first antibody** used so that it will not bind any subsequent primary antibodies. When method 1 is included in the

staining protocol, it is equally important to stain cells using method 1 followed by the block and a directly conjugated isotype control immunoglobulin. If the isotype control binds to any of the cells stained with the primary/secondary combination, the block has not been effective and the data will be artifactual.

Dead cells can bind antibodies nonspecifically leading to an erroneous percentage of positive cells for some antibodies. It is important to account for these dead cells because antibodies bind to them nonspecifically.

EMA will photolabel the DNA of nonviable cells when exposed to visible light (white light). These cells can then be fixed and the EMA, if incorporated, will remain bound to the DNA. The procedure follows:

1. Add 50 µl of cells to a 12 x 75 mm tube, wash. If erythrocyte lysis is desired, lyse cells with lysing reagent centrifuge, decant and blot tube.
2. Add 8 µl of EMA (5 µg/ml) and put the tube 18 inches beneath a fluorescent light for 10 mins. (Lay flat in white pan). Wash.
3. Resuspend cells in 0.5 ml of 2% ultrapure formaldehyde. [If it is desired to analyze viable cells, substitute the desired resuspension medium].

EMA may stain viable cells slightly causing some interference with setting the marker if combined with other antibodies. We recommend using a separate tube for viability assessment and backgating to locate the live and dead cells in the FSC vs SSC view. The gate on viable cells is then applied to the analysis of subsequent antibody stained samples.

Reference: Reidy, M. C., Muirhead, K. A.,

Jensen, C. P., and Stewart, C. C. The Use Of A Photolabeling Technique To Identify Nonviable Cells In Fixed Homogenous Or Heterologous Cell Populations. Cytometry 12:133-139, 1991

## Data Acquisition

We first need to define parameters. This is a generic term used to describe the entities we will measure. There are three common kinds of parameters: forward scatter (FSC) and side scatter, (SSC), which is also called orthogonal or 90° scatter, because it is measured at 90 degrees to the laser beam and fluorescence. The FSC is proportional to the cross sectional area of the particle and provides a measurement of size, while SSC is sensitive to cell granularity. The intensity of fluorescence is also measured orthogonal to the cell stream.

The five commonly used fluorochromes that are conjugated to second antibodies, avidin or to monoclonal antibodies are fluorescein isothiocyanate (FITC), phycoerythrin (PE), a phycoerythrin-texas red tandem complex (PETR), a phycoerythrin-cyanin 5 tandem complex (PECy5) and peridinin chlorophyll-P (PerCP). These fluorochromes can each be excited at 488 nM and the fluorescence emission from FITC = 530 nM, from PE = 575 nM, from PETR = 613 nM and from both PECy5 and PerCP = 670 nM.

Up to four fluorescence antibodies can be combined because the colors can be separately measured, but we will consider only three colors. To accomplish color separation, spectral filters and dichroic mirrors are used that reflect or transmit specific colors. Because the fluorochrome's emission wavelengths can be very broad, it is also necessary to remove unwanted overlapping colors by electronic compensation. This is accomplished by appropriately adjusting the instrument's compensation circuits. To perform this task it is necessary to use compensation standards. These standards can be purchased if only FITC or PE fluorochromes are to be used, but if the other colors are also desired, compensation standards must be prepared by the user.

To prepare these standards for up to three separate colors, 50 µl of blood is placed in four separate tubes. Only cells are added to the first tube, and this is used to adjust the high voltage. To the second tube add PE-CD4, to the third tube add FITC-CD45, and to the last tube add PECy5-CD45 (a PETR or PerCP antibody could be used instead).

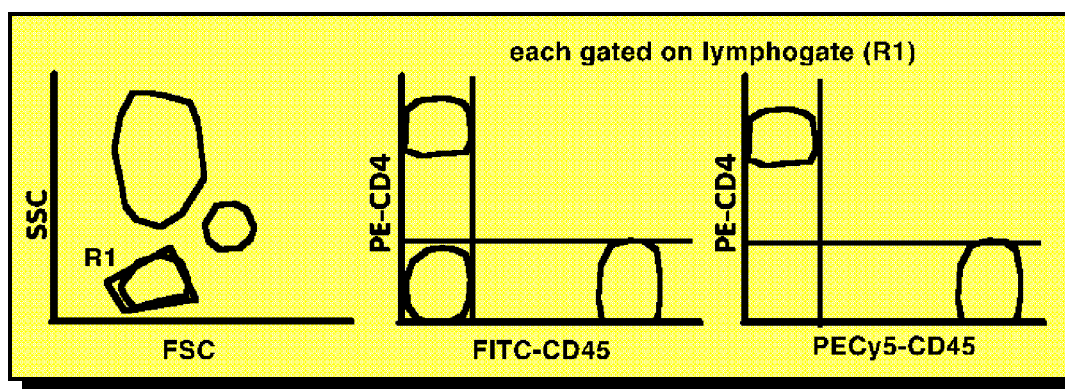


Figure 1: Color Compensation for Three Color Data Acquisition

15 minutes incubation, on ice, wash, lyse erythrocytes and fix the cells. Only cells stained with the antibodies for the desired

fluorochromes need to be prepared and used for compensation.

A properly compensated instrument for four color data acquisition is shown in **Figure 1**. All five tubes are superimposed in this

single figure as if the cells had been mixed together in one tube after staining them with the antibodies. The FSC vs SSC is displayed and a lymphocyte gate (R1) is produced.

**TABLE 1: CORRELATED LIST MODE FILE**  
*Values for each parameter*

Cell	P1	P2	P3	Pn
C1	122	84	20	440
C2	424	92	20	460
C3	86	83	562	22
C4	101	98	487	33
Cn	330	79	30	452

are plotted against the measured values (on the x axis or abscissa). We need an objective way to tell positive from negative and there are two popular methods. The first is to

Using the high voltage adjustments, the unstained cells in tube one are centered in the lower left corner of each bivariate plot. Taking each tube beginning with the first, each compensation is adjusted so that cells labeled with the appropriate fluorochrome/antibody are in the correct quadrant. The instrument is now ready to acquire data derived from patient specimens.

acquire data from cells that have been procedurally treated the same as those receiving antibody but do not actually receive any antibody. As shown in **Figure 2A**, these cells provide a minimum fluorescence value and a marker can be created above which cells are called positive. This is called an autofluorescence distribution and the marker is set so that usually, no more than 0.5 to 2% of

## Data Analysis

All flow cytometers collect data in the same way using a process called **LIST MODE**. A standard list mode file includes a header followed by a list of data in the order collected. The values that are stored in a list mode file are the voltages from the detectors for each parameter (P1, P2, P3,... Pn) after they have been electronically processed. As shown in **Table 1**, the data in a list mode file, consists of the voltage for each parameter for cell #1 followed by cell #2 and so forth until the last cell, n, is measured. If six parameters are measured for 10,000 cells, the file will contain 60,000 numbers in the order they were acquired.

The univariate histogram shown in **Figure 2** is the simplest of all ways to display data. It is a frequency distribution where the number of events (on the y axis or ordinate)

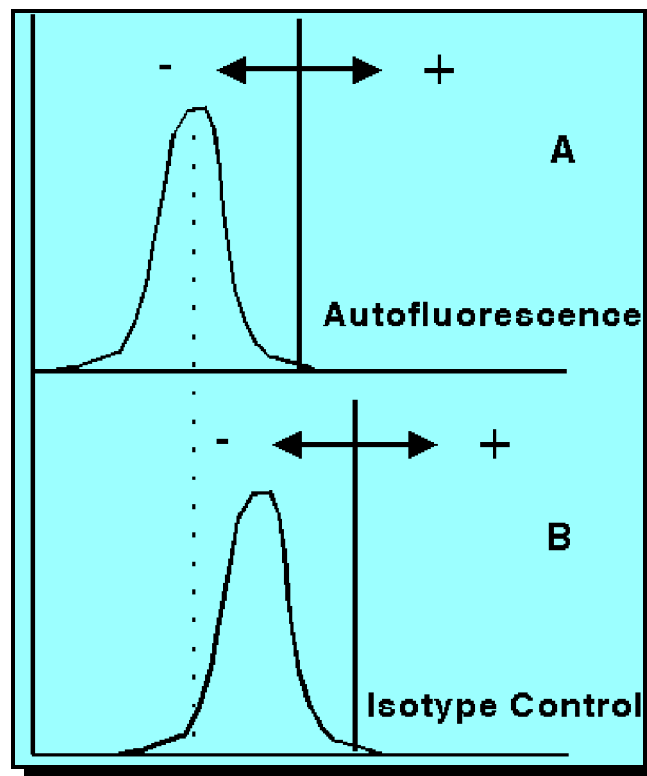


Figure 2: Setting Markers in a Univariate Histogram

cells are above it. The second method is to use an **isotype** control immunoglobulin as discussed above. As shown in **Figure 2B** the

we restrict the values of interest to a region that we wish to know about. Only cells that meet the region's criteria are evaluated.

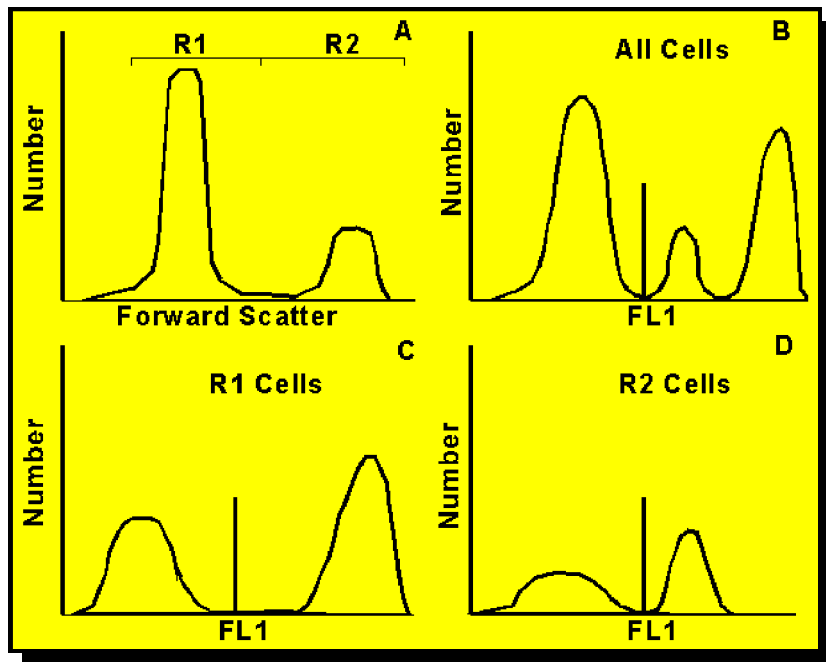


Figure 3: Gating Histogram

cells may be somewhat brighter than the autofluorescence because of non-specific and Fc receptor binding. Blocking can reduce this component significantly.

Again, the user must decide what marker position will be used to distinguish positive from negative cells and a position between 0.5% to 2% of cells above the marker is customary.

Using the univariate histogram, we can analyze a list mode data file. In **Figure 3A**, we have defined two regions: R1 for small particles and R2 for large particles in the FSC histogram. We could find out the percentage of cells that are positive in R1 or R2 because the data analysis program would sequence through every FSC value in the list between the selected values. We could then display them in the fluorescence histograms for all cells (B) or for those in R1 (C) or for those in R2 (D). We term this process **gating** because

As shown in **Figure 3C**, there are two distinct frequency distributions similar in position to the same negative cells and all the bright cells in **Figure 3B**. The ones on the left of the marker exhibit low fluorescence while the ones on the right exhibit high fluorescence so it is easy to tell the cells that are positive from those that are negative. In D, some of the negative cells are also found, but so is the dimmer positive population.

When one univariate histogram is plotted on the x axis and a second univariate histogram is plotted on the y axis as shown in **Figure 4**, a bivariate histogram results. If the two univariate histograms from **Figure 2** are each projected into the area of overlap, the values in common of FSC and FL1 produce clusters of the cells represented by the circles.

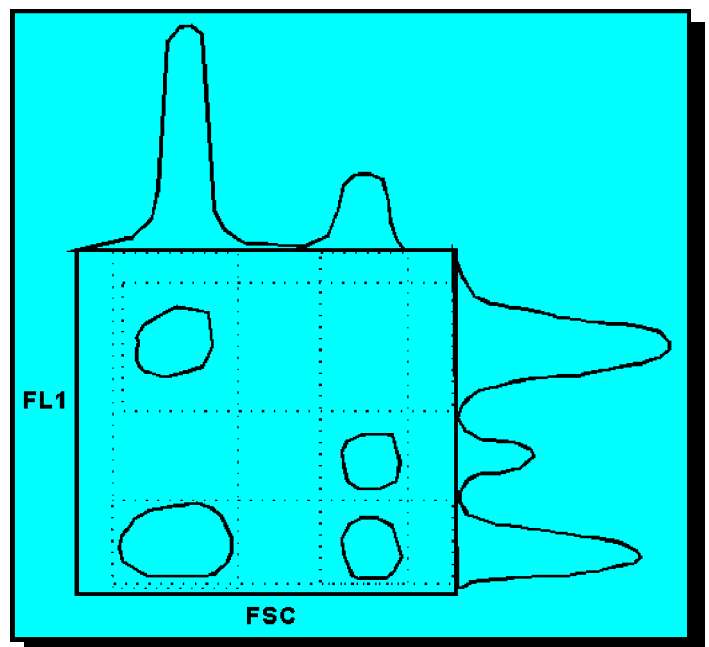


Figure 4: Bivariate Histogram

These clusters can be of any size and shape depending upon the relationship between the two properties that were measured.

In order to make analysis easier when bivariate plots of data are used, quadrant markers are used. These markers are set along the x and y axis, as shown in **Figure 5**, to produce quadrants so that all combinations of + and - cells can be displayed. For two antibodies, there are four possible combinations, +-, ++, -+ and --.

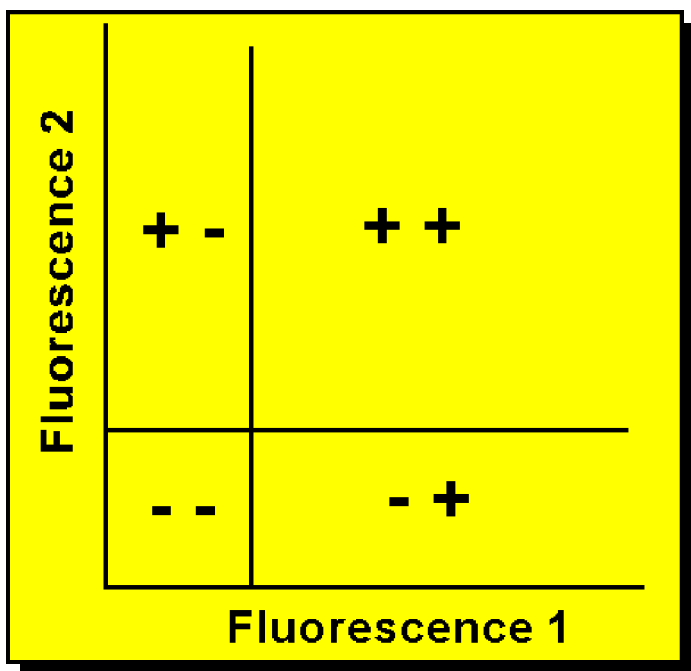


Figure 5: Setting Quadrant Markers

As shown in **Figure 6**, a region (R1) can be drawn in the FSC vs SSC display (**Figure 6A**) to include only the small cells with low SSC. In this example blood was stained with CD4 and CD8 and the small cells are lymphocytes. This region is used as a gate to display their fluorescence in a second bivariate display. Using an isotype control, the quadrant markers are set in **Figure 6B** so that 1% of cells are above them. In **Figure 6C**, CD4 and CD8 are shown and the percentage of positive events in each category can be obtained.

One problem associated with gating cells on the basis of size is an assumption that there are no cells of interest outside the selected region. In the above example, lymphocytes were selected but are all the lymphocytes in the region selected and secondly are all the cells in the region selected lymphocytes? For example, the cells colored blue (CD4+) that are below the major blue cluster are not lymphocytes; they are monocytes in the lymphogated region. It is possible to answer the two questions quite objectively by using CD45 that labels all leukocytes and CD14 that labels only monocytes.

The purity of the region in the FSC vs SSC display for lymphocytes and the proportion of them within the gate can be evaluated. To accomplish this, all cells are

We can now combine gating and quadrant analysis to analyze 4 parameter flow cytometry data consisting of FSC, SSC and two colors of fluorescence.

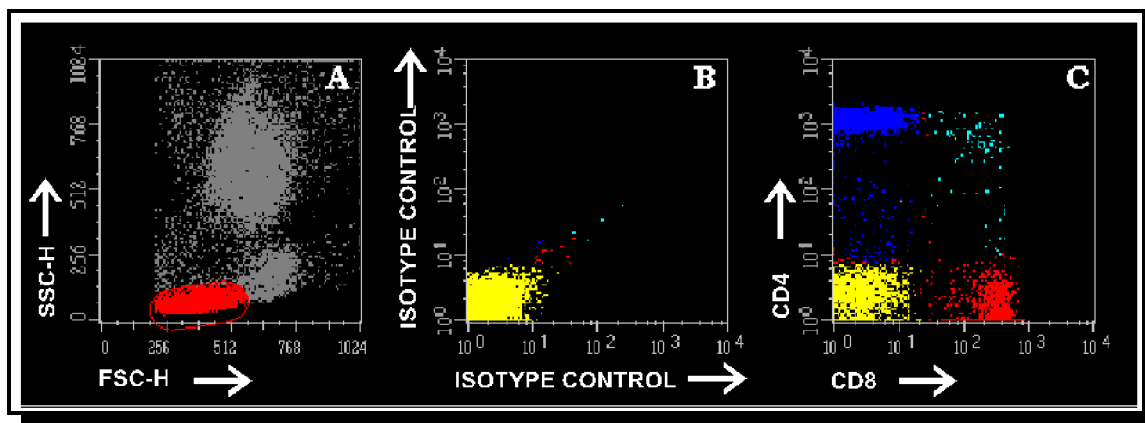


Figure 6: Gating a Selected Population



acquired and their CD45 vs CD14 distributions displayed as shown in **Figure 7** for blood. A region R2 containing all viable lymphocytes is drawn around the CD45 bright CD14<sup>-</sup> cells. This region is applied as a gate to the FSC vs SSC display and the location of all lymphocytes is found. A second region, R1, is drawn around the lymphocytes and their percentage recorded.

If this region

is drawn large enough, it will contain undesired cells so lymphocyte purity is reduced. To evaluate the purity, this gate is applied to the CD45

CD14 bivariate display and the percentage of cells in R2 representing the lymphocytes is recorded. As shown in the lower right display, green dots are lymphocytes and red dots are contaminating cells, so the purity is only 92.5%. If the purity of the lymphogate is low, it may be improved by reducing the size of R1. This will cause a loss of lymphocytes while improving purity. As long as the loss of only a small proportion of lymphocytes occurs concomitantly with a significant improvement in purity, the procedure can be used to optimize the lymphogate. This gate, once established, is then applied to the analysis of all subsequent samples. This same strategy can

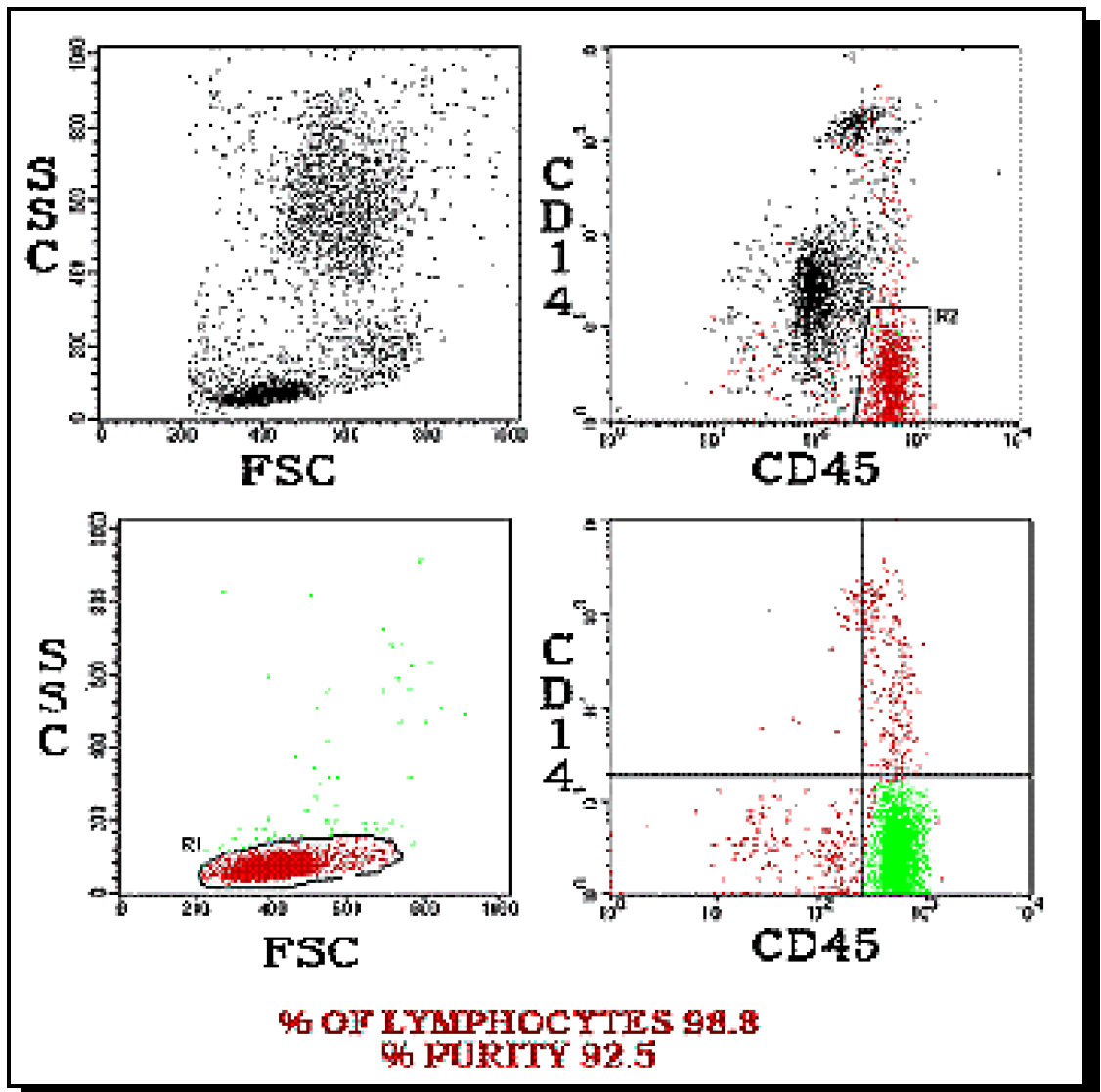


Figure 7: Quality of Gate

be applied to the monocyte and granulocyte regions.

### Three Color Immunophenotyping:

When a third color is introduced, there are three views for displaying all the fluorescence data, shown in **Figure 8**. They are FL1 vs FL2, FL1 vs FL3, and FL2 vs FL3. Since there are three plots, we need to be able to visualize the position of cells in one view with the same cells in another. This can be accomplished by using color.

A population of cells in FL1 vs FL2 has been colored red. These cells could be anywhere along the projection of FL2 onto the FL3 axis or FL1 on to the FL3 axis.

As shown in **Figure 8A**, a single population is seen, but in **Figure 8B**, two distinct populations, one that is FL3<sup>-</sup> and one that is FL3<sup>+</sup> are found. Since we colored the cells red, both populations resolved in FL3 would also be red, even though one of them is FL2<sup>+</sup>FL3<sup>-</sup> and the other is FL2<sup>+</sup>FL3<sup>+</sup>. This situation can be corrected by coloring the FL2<sup>+</sup>FL3<sup>+</sup> population green. In **Figure 8C** we

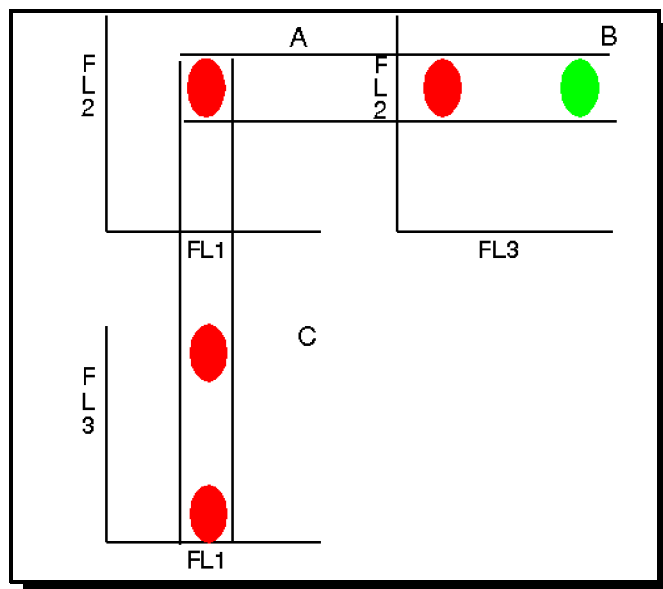


Figure 8: Displaying Three Parameters

three bivariate displays need to have quadrants because that will produce all the possible combinations for the three fluorescent antibodies. Any two can be used and in this example FL1 vs FL2 and FL3 vs FL2 was chosen. Based on whether any given cell is positive or negative for any given antibody, there are eight possible binary combinations, shown in **Table 2**.

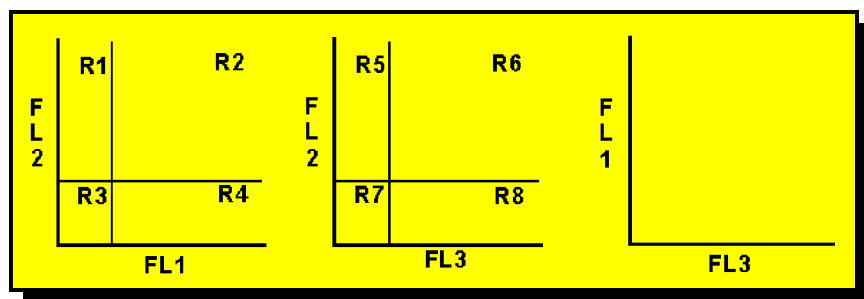


Figure 9: Combining Quadrant Markers

are able to see that both populations are also FL1<sup>+</sup>.

For the automatic analysis of large numbers of specimens, this method of coloring is time consuming. A more streamlined strategy can be developed using the quadrant markers shown in **Figure 9**. Each quadrant is assigned a region, R1-R8. Only two of the

Cells with any given phenotype can, therefore, be explicitly defined by combining the regions using boolean algebra. Cells that are not stained can only be found in R3 and R7. We cannot use R3 alone because they could also be found in either R7 or R8. Similarly, we cannot use R7 alone because they could be found in

either R3 or R4. Thus, R3 and R7 explicitly defines the only location cells negative for all three antibodies can be found. This same logic is used for all the other seven possible combinations.

The next step is to assign each of these combinations specific colors. Each color will correspond to a specific positive and negative

combination of antibodies it was assigned regardless of the antibody combinations used. Each combination of different antibodies will produce its own unique colored pattern as shown for the panel FITC-CD3, PE-CD4, and TC-CD8 in **Figure 10**.

In **Figure 10**, a lymphogate was first established using the method shown in **Figure 7** and using the binary combinations shown in **Table 2**. FITC-CD3 is FL1, PE-CD4 is FL2 and PerCP-CD8 is FL3. Compare this with **Figure 6** where the CD4 dim cells (blue in **Figure 6**), are actually CD3 negative (green in **Figure 10**).

For non-malignant cells, these patterns will always be the same because the

molecules (cellular differentiation antigens) on the cell's membrane that bind the specific antibodies for them are highly regulated. This is not true for malignant cells which exhibit altered expression of some, but not all, antigens. Each person's leukemia or lymphoma is unique and no two are exactly alike. This does not mean, however, that there aren't general categories into which different hematopoietic malignancies fall. These

categories fall into lineage assignments that share some properties in common with normal cells of the same differentiation stage. An example for a T-lineage acute lymphocytic leukemia is shown in **Figure 11**. Most T-ALL

**TABLE 2:  
BINARY COMBINATIONS OF QUADRANT MARKERS.**

FL1	FL2	FL3	
-	-	-	R3 and R7
+	-	-	R4 and R7
-	+	-	R1 and R5
+	+	-	R2 and R5
-	-	+	R3 and R8
+	-	+	R4 and R8
-	+	+	R1 and R6
+	+	+	R2 and R6

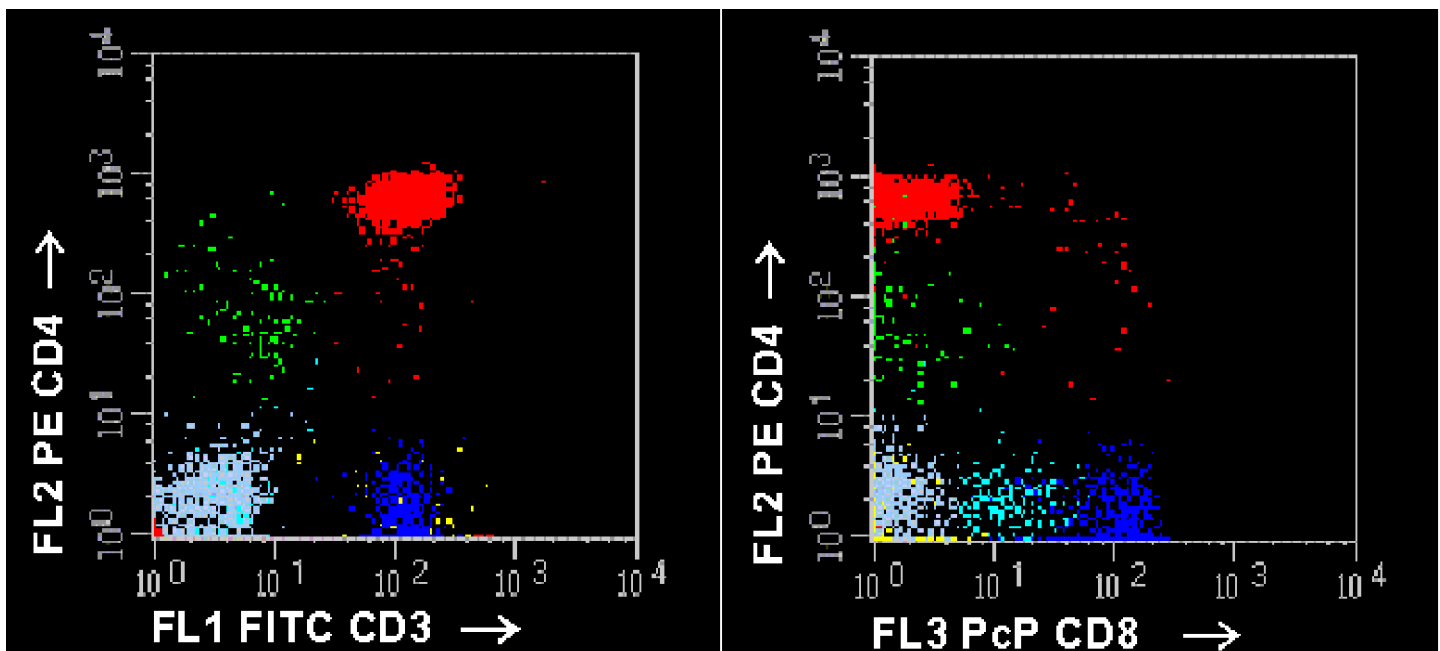


Figure 10: Normal T Cells

cells are colored red but in comparison with **Figure 10**, the normal T-cell pattern can also be seen.

processing, data acquisition and analysis and the professional component which consists of a written interpretation of the data. The professional interpretation is performed by a certified individual that does not have to be a physician. The degree of interpretation depends on the test. For example, an HIV

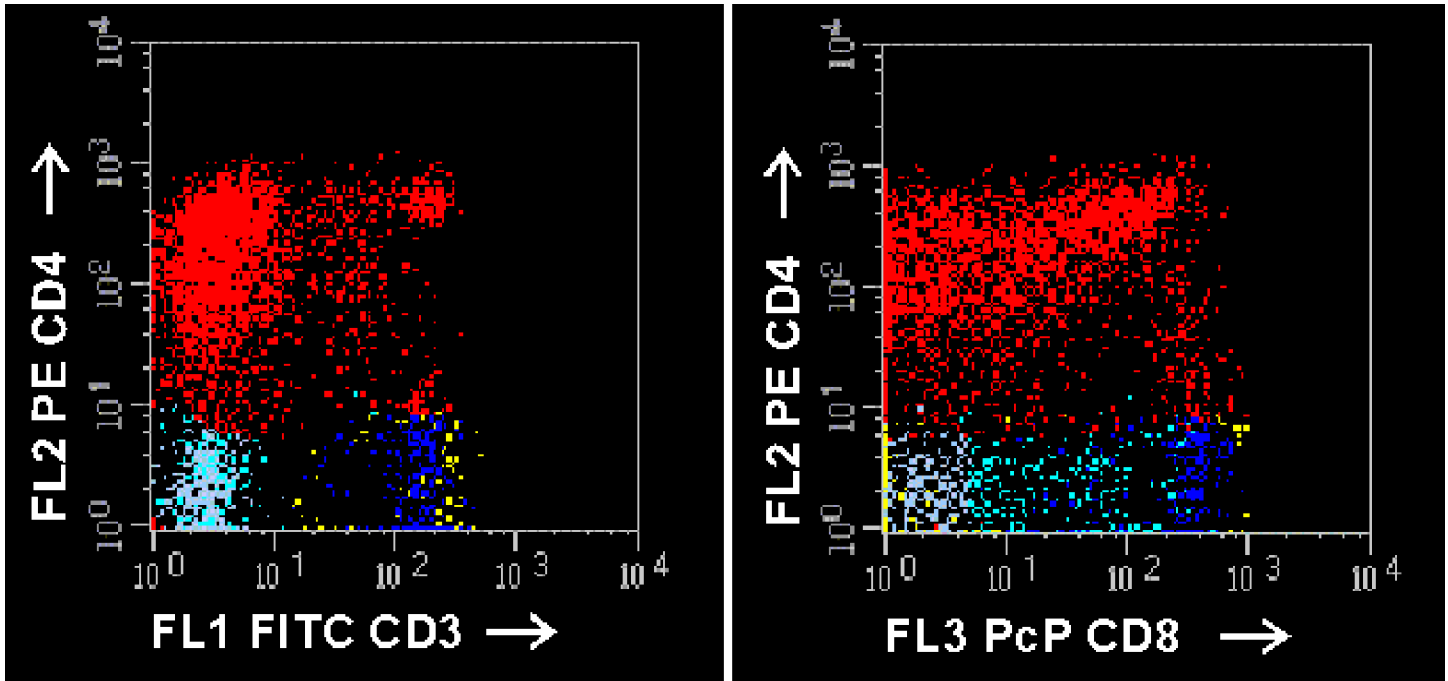


Figure 11: T Cell Acute Lymphocytic Leukemia

### The Clinical Immunophenotyping Report

There are two kinds of clinical tests currently recognized by the medical reimbursement community. The first is called a clinical laboratory test (CLT). The second is called a clinical anatomical test (CAT). The major distinction between these two tests is the second requires an interpretation by a professional, while the first provides the clinician with numerical values only. Flow Cytometry tests are considered clinical anatomical tests because an interpretation is often, but not always, required. This test can be divided into two additional components: a technical component that consists of sample

screen that consists only of a CD3<sup>+</sup> CD4<sup>+</sup> count requires no interpretation and is like a clinical laboratory test. In contrast, a leukemia screen will require the use of several antibodies in combination to produce patterns that need to be interpreted. In this case the use of percentages in the report is of less value for the clinician who would rather know the diagnosis and classification of the leukemia or lymphoma, i.e. the interpretation. Suggested antibody combinations are shown in **Table 3**.

TABLE 3. SUGGESTED ANTIBODY COMBINATIONS FOR IMMUNOPHENOTYPING

Immunodeficiency	Lymphocytic Malignancy	Myeloid Malignancy
FL-CD45	FL-CD45	FL-CD45
PE-CD14	PE-CD14	PE-CD14
PP-HLADr	PP-HLADr	PP-HLADr
FL-CD3	FL-CD3	FL-CD3
PE-CD4	PE-CD4	PE-CD4
PP-CD8	PP-CD8	PP-CD8
FL-CD16	FL-CD5	FL-CD33
PE-CD56	PE-CD19	PE-CD13
PP-CD3	TC-CD10	PP-HLADr
	FL-CD20	FL-CD16
	PE-CD11c	PE-CD32
	TC-CD22	TC-CD64
	FL-KAPPA	FL-CD11b
	PE-LAMBDA	PE-CD13
	TC-CD19	TC-CD33
	FL-IgD	FL-CD38
	PE-IgM	PE-CD34
	TC-CD19	PP-HLADr
	FL-CD5	FL-CD15
	PE-CD34	PE-CD34
	TC-CD19	TC-CD56

FL = FITC, PE = phycoerythrin, TC = PE-Cy5 tandem, PP = PerCP

Isotype Control containing FL-IgG1, PE-IgG2b and TC-IgG2a, autofluorescence sample and EMA sample included in every screen

The online discussion for this presentation can be found in Conference Area #60, on Med TechNet; and will be active from October 23 through November 12, 1995. Successful completion of the online post-quiz earns the participant 3.0 P.A.C.E.® CEU's.

This is a presentation of Med TechNet Online Services for the Clinical Laboratory. "Talks", such as this one, last for three weeks, during which time, the author and other participants discuss the topic. Users can participate by telnet'ing to Med TechNet on the Internet at, [bbs.medtechnet.com](http://bbs.medtechnet.com), or dialing in directly, via modem, at, 1-716-688-1552, or, 1-716-636-8235. Discussions can also be distributed via Internet E-Mail.

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