C.C. Stewart, S.J. Stewart, Titering Antibodies. In: Current Protocols in Cytometry, (J.P. Robinson, Z. Darzynkiewicz, P. Dean. L. Dressler, P.Rabinovitch, C. Stewart, H. Tanke, L. Wheeless, eds.) J.Wiley & Sons, Inc., New York, 4.1.1 - 4.1.13, 1997.

Titering Antibodies

INTRODUCTION

Monoclonal antibodies are the primary kind used for immunophenotyping. These antibodies are almost always derived from the immunization of mice with the appropriate antigen. They are almost always of the IgG isotype. the most frequent subclasses are IgG1, IgG2a and IgG2b. IgM is a less common isotype of another monoclonal antibodies, but, because it is approximately five times the molecular weight of IgG, it may be more troublesome/ Polyclonal antibodies derived from serum of immunized animals is less frequently used as the primary antibody directed to the desired antigen, but is almost always used as the second fluorochrome conjugated antibody. The F(ab)'$_2$ fragment should always be used to minimize Fc receptor binding of polyclonal antibodies. F(ab)'$_2$ fragments of monoclonal antibodies are not readily available because active rodent derived F(ab)'$_2$ fragments are difficult to prepare in high yield.

In this unit we will define the strategy for titering antibodies that will give the highest discrimination of positive cells from negative cells. Once their titer has been defined, the antibodies may be combined for providing simultaneous evaluation of multiple antigens expressed by cells.

Materials

target cells for antibody to be titered
PBS Phosphate buffered saline without Calcium or Magnesium (Gibco-BRL #21300-082))
normal mouse IgG (Caltag #10400)
normal goat IgG (Caltag #10200)
12 x 75 mm polypropylene test tubes (Falcon 2052)
ultrapure formaldehyde (Polysciences #04018)
lysing solution (see recipe)
Sorvall centrifuge rotor H1000B or equivalent
eppendorf centrifuge, airfuge or equivalent
antibodies
isotype control myeloma proteins
flow cytometer

Procedure for titering antibodies to extracellular antigens

A. Directly conjugated antibodies

1. Determine the concentration of antibody in the stock solution and centrifuge it at 15,000 x g. Leave aggregated antibody in pellet.
2. Prepare 30 μl containing 9 μg of Ab (300 μg/ml) in PBS. Prepare six serial 10 μl to 30 μl (1/3) dilutions.

3. Prepare a target cell suspension containing 5 - 10 x 10^6 cells/ml and containing 200 μg normal mouse IgG/ml.
4. Add 10 μl of each antibody dilution to 50 μl of cells in separate test tubes. Also prepare an isotype control tube and a tube containing only cells.
5. Incubate 15 minutes and then add 3 ml lysing solution.
6. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant, blot tube lip on absorbent towel and resuspend cells in residual solution.
7. Add 200 μl 2% ultrapure formaldehyde.
8. Acquire data using a flow cytometer.

B. Biotinylated or hapten conjugated antibodies

1. Determine the concentration of antibody in the stock solution and centrifuge it at 15,000 x g. Leave aggregated antibody in pellet.
2. Prepare 30 μl containing 9 μg of Ab (300 μg/ml) in PBS. Prepare six serial 10 μl to 30 μl (1/3) dilutions.
3. Prepare a target cell suspension containing 5 - 10 x 10^6 cells/ml and containing 200 μg normal mouse IgG/ml.
4. Add 10 μl of each antibody dilution to 50 μl of cells in separate test tubes. Also prepare an isotype control tube and a tube containing only cells.
5. Incubate 15 minutes and then add 3 ml lysing solution.
6. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant, blot tube lip on absorbent towel and resuspend cells in residual solution.
7. Add 10 μl of appropriately titered avidin or anti-hapten antibody with associated conjugated fluorochrome.
8. Incubate 15 minutes and then add 3 ml PBS.
9. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant and blot tube lip on absorbent towel and resuspend cells in residual solution.
10. Add 200 μl 2% ultrapure formaldehyde.
11. Acquire data using a flow cytometer.

C. Neat antibody

1. Determine the concentration of antibody in the stock solution and centrifuge at 15,000 x g. Leave aggregated antibody in pellet.
2. Prepare 30 μl containing 9 μg of Ab (300 μg/ml) in PBS. Prepare six serial 10 μl to 30 μl (1/3) dilutions.
3. Prepare a target cell suspension containing 5 - 10 x 10^6 cells/ml and containing 200 μg IgG of the second antibody species, e.g. goat. Do not use normal mouse IgG.
4. Add 10 μl of each antibody dilution to 50 μl of cells in separate test tubes. Also prepare an isotype control tube and a tube containing only cells.
5. Incubate 15 minutes and then add 3 ml lysing solution.
6. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant, blot tube lip on absorbent towel and resuspend cells in residual solution.
7. Add 10 μl of appropriately titered fluorochrome conjugated second antibody.
8. Incubate 15 minutes and then add 3 ml PBS.
9. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant, blot tube lip on absorbent towel and resuspend cells in residual solution.

10. Add 200 μl 2% ultrapure formaldehyde.
11. Acquire data using a flow cytometer.

Data Analysis
1. Display a histogram for each dilution, as shown in figure 1.
2. Adjust markers using auto sample (or isotype control sample) so that less than 1% of events are above the marker.
3. Determine the mean channel linear fluorescence intensity (MCF) of both positive (Signal) and negative (Noise) cells for the six tubes.

Compute the signal to noise ratio by dividing the mean channel fluorescence (MCF) value for positive cells by that for the negative cells and plot these values as a function of antibody dilution as shown in figure 2. The highest ratio is the optimal titer because this value provides the greatest discrimination between positive and negative cells, regardless of the absolute value of fluorescence intensity.

Procedure for intracellular antigens
Materials
     target cells for antibody to be titered
     PBS Phosphate buffered saline without Calcium or Magnesium (Gibco-BRL #21300-082))
     normal mouse IgG (Caltag #10400)
     normal goat IgG (Caltag #10200)
     12 x 75 mm polypropylene test tubes (Falcon 2052)
     ultrapure formaldehyde (Polysciences #04018)
     lysing solution (see recipe)
     Sorvall centrifuge rotor H1000B or equivalent
     eppendorf centrifuge, airfuge or equivalent
     antibodies
     isotype control myeloma proteins
     flow cytometer

A. Directly conjugated antibodies
There is no universal method for fixing and permeabilizing all types of cells so some adjustments may be required to the recommended starting procedure. There are several excellent commercially available fixatives and permeabilizing solutions for this purpose. Even more serious is the consideration that enzymes, like trypsin, that are often used to disperse cells may also strip the desired antigen epitopes. To further complicate matters, fixation may also destroy the epitope. The freeze-thaw procedure has the advantage that epitopes are not distorted. The major problem with this procedure is cell loss. It is performed by rapid freezing cells in an alcohol dry ice bath and thawing them at 37° for 3 to 5 cycles.
1. Determine the concentration of antibody in the stock solution and centrifuge at 15,000 x g. Leave aggregated antibody in pellet.
2. Determine the concentration of an isotype control immunoglobulin and centrifuge at 15,000 x g. Leave aggregated immunoglobulin in pellet.

3. Prepare 30 μl of antibody and 30 μl of isotype control each containing 9 μg (300 μg/ml) in PBS. Prepare six serial 10 μl to 30 μl (1/3) dilutions.

4. Prepare fixed permeabilized target cells that express the desired protein and fixed permeabilized target cells that do not express the desired protein. Both cell suspensions should be at 5 - 10 x 10^6 cells/ml.

5. Add 10 μl of each antibody dilution and 10 μl of each isotype control dilution to 50 μl of each target cell in the appropriate tubes. Also prepare tubes containing each cell type alone.

6. Incubate cells 45 min. and then add 3 ml of PBS (or lysing solution), incubate an additional 45 min., centrifuge cells 3 min. at 1500 x g and discard the supernatant. Resuspend cells in residual solution.

7. Acquire data using a flow cytometer.

B. Biotinylated or hapten conjugated antibodies or neat antibodies

1. Determine the concentration of antibody in the stock solution and centrifuge at 15,000 x g. Leave aggregated antibody in pellet.

2. Determine the concentration of an isotype control immunoglobulin and centrifuge at 15,000 x g. Leave aggregated immunoglobulin in pellet.

3. Prepare 30 μl of antibody and 30 μl of isotype control each containing 9 μg (300 μg/ml) in PBS. Prepare six serial 10 μl to 30 μl (1/3) dilutions.

4. Prepare fixed permeabilized target cells that express the desired protein and fixed permeabilized target cells that do not express the desired protein. Both cell suspensions should be at 5 - 10 x 10^6 cells/ml.

5. Add 10 μl of each antibody dilution and 10 μl of each isotype control dilution to 50 μl of each target cell in the appropriate tubes. Also prepare tubes containing each cell type stained with the second reagent as well as unstained cells. Incubate the cells 45 min.

6. Add 3 ml of PBS (or lysing solution), incubate an additional 45 min., centrifuge cells 3 min. at 1500 x g and discard the supernatant. Resuspend cells in residual solution.

7. Add 10 μl of appropriately titered avidin or anti-hapten antibody or second antibody with associated conjugated fluorochrome.

8. Incubate cells 45 min. and then add 3 ml of PBS, incubate an additional 45 min., centrifuge cells 3 min. at 1500 x g and discard the supernatant. Resuspend cells in residual solution.
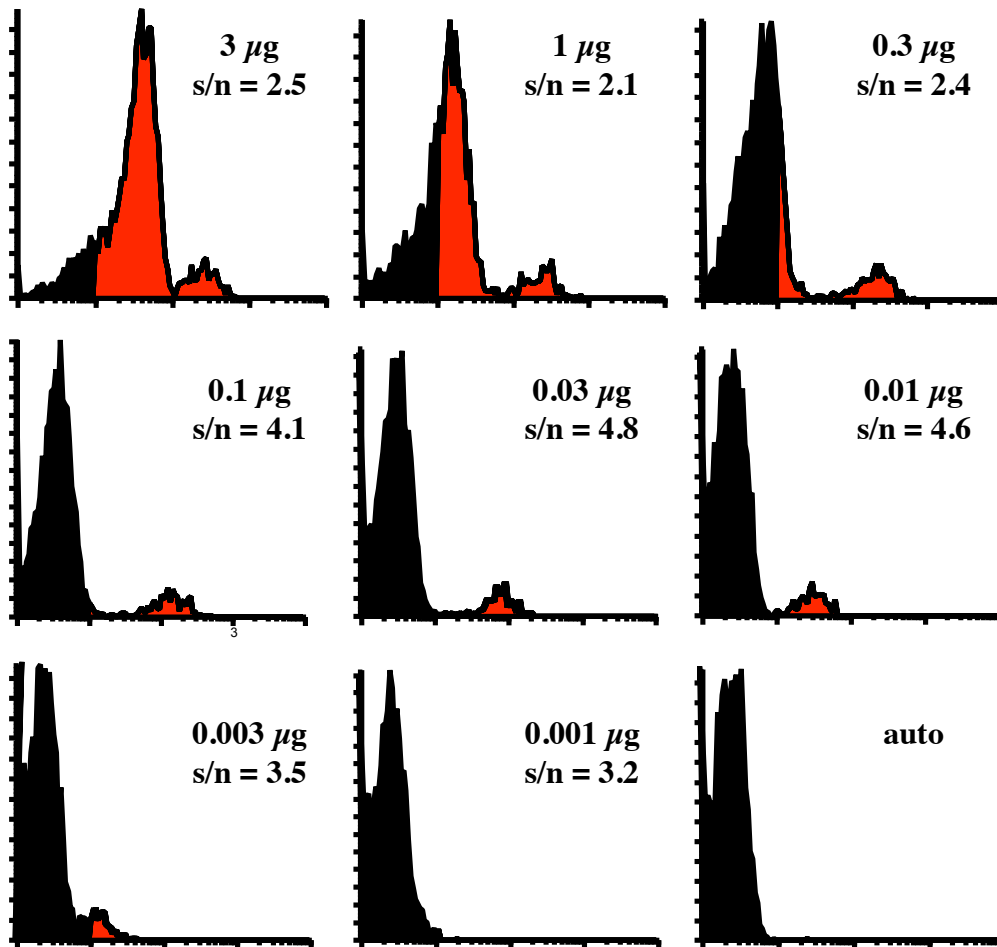
9. Acquire data using a flow cytometer.
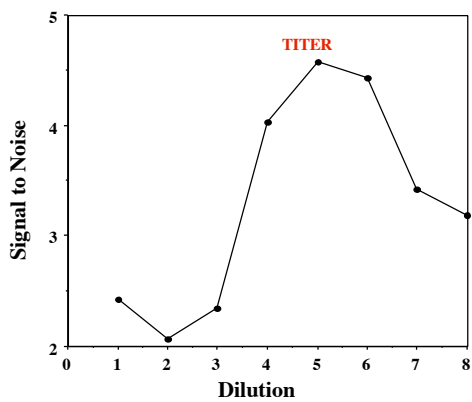
Figure 1. Determining antibody titer.

Figure 2. Titer at Maximum Signal to Noise Ratio

Data Analysis

1. Display histogram for each dilution as shown in figure 3 for antibody and isotype control for both positive and negative cells.
2. Determine the MCF for each.

3. Determine the ratio of the MCF for antibody to MCF for isotype control and plot these
    values as a function of dilution, as shown in figure 4.

1 μg S/N
Ab   278
IC   5.8

1 μg S/N
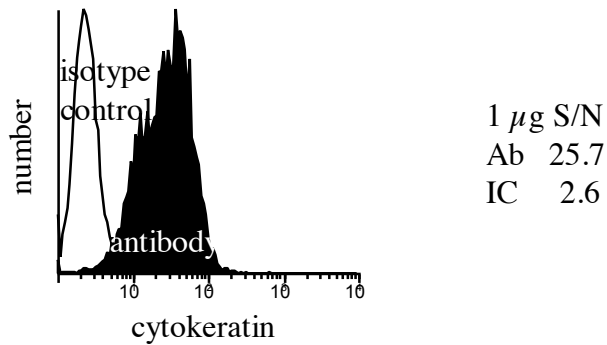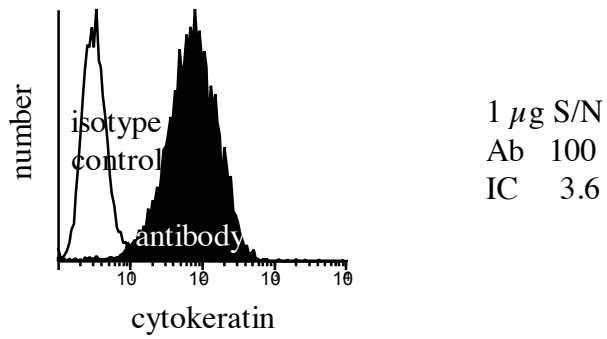Ab   100
IC   3.6

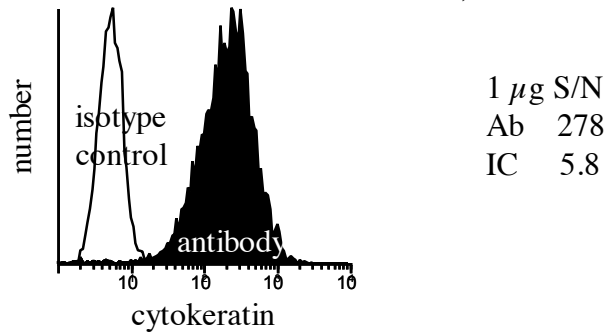1 μg S/N
Ab   25.7
IC   2.6

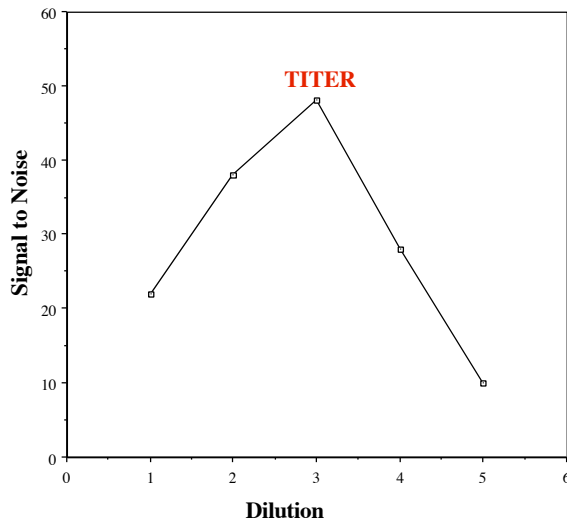Figure 3. Determining Titer of Antibody to Intracellular Antigens

Figure 4. Titer at Maximum Signal to Noise Ratio for Intracellular Antigens (Dilutions 1 and 2 are not shown in Figure 3)

A usable antibody is one that has a S/N >3.0 for positive cells. Sometimes it is not possible to find an antibody concentration that meets the above specifications. This can be due to an inappropriate fluorochrome because the epitope density on the target cell is too low for good resolution of positives. The problem can be corrected by using a brighter fluorochrome such as PE. Alternatively, a second reagent might be used to improve fluorescence but this approach can also increase noise. If, however, these approaches fail to improve the S/N, it may be the antibody itself is of poor quality and finding an alternative source may be warranted.

Epitope Titer

The previous procedure is designed to determine the antibody titer at a constant epitope concentration. Clearly, the titer of any antibody is also dependent upon the concentration of epitopes. The concentration required to saturate cells with 10^5 epitopes would be different that that required to saturate cells with 10^4 epitopes and both types might be present in the same suspension. To determine this titer, the antibody concentration is held constant and the epitope concentration is varied by increasing the cell concentration.

1. Adjust target cells to 500 x 10^6 /ml and prepare 4 serial 1/4 dilutions to produce 64, 16, 4 and 1 x 10^6 cells in 50 μl of PBS. If blood or bone marrow leukocytes are to be used as target cells: put 5 ml in a 50 ml centrifuge tube containing 45 ml lysing reagent and centrifuge at 1500 x g for five minutes. Remove the supernatant and resuspend cells in residual solution.
2. Mix 10 μl of antibody whose titer was determined using 0.5 - 1 x 10^6 cells with 50 μl of cells.
3. Incubate 15 minutes and then add 3 ml PBS.
4. Centrifuge tubes at 1500 x g for three minutes. Decant the supernatant, blot tube lip on absorbent towel and resuspend cells in residual solution.

5. Add 200 μl (or more, depending on starting cell concentration) of 2% ultrapure formaldehyde.
6. Acquire data using a flow cytometer.

Data Analysis
1. Display histogram for each dilution.
2. Adjust markers using auto sample (or isotype control sample) so that less than 1% of events are above the marker.
3. Determine the mean channel linear fluorescence intensity (MCF) of both positive (Signal) and negative (Noise) cells for the four tubes.

The expression of any particular epitope on a population of cells can vary by an order of magnitude. For example, the epitope number for CD19 molecules is an order of magnitude less than the epitope frequency for CD20 on B-cells. Similarly, the epitope frequency of CD4 on T-cells is about five times greater than on monocytes. The epitope concentration, therefore is equal to the average number of molecules per cell times the number of cells divided by the volume. It is important to know the epitope titer if scaling up the staining of target cells is desired. For example, if the antibody titer is known for 10^6 cells/ml, 100 times this amount may not be required to stain 10^8 cells/ml. Because of the cost of antibodies, it is important not to use more than necessary.

Antibody Combinations

When antibodies are purchased as single reagents with the intent to combine them for clinical immunophenotyping, it is necessary for the clinical laboratory to verify their performance. Each combination must prove to stain properly and each prepared batch must provide the same performance as subsequent batches. The laboratory must also show the intended use of the combination is efficacious. We recommend using only antibodies with directly conjugated fluorochromes. To determine if antibodies perform properly when combined, a simple test is performed.

Materials
target cells for combination to be titered
PBS Phosphate buffered saline without Calcium or Magnesium (Gibco-BRL #21300-082))
normal mouse IgG (Caltag #10400)
12 x 75 mm polypropylene test tubes (Falcon 2052)
ultrapure formaldehyde (Polysciences #04018)
lysing solution (see recipe)
Sorvall centrifuge rotor H1000B or equivalent
antibodies with appropriate fluorochromes
isotype control myeloma proteins with appropriate fluorochromes
flow cytometer

Procedure
1. Using each antibody at its proper titer, add each one to separate test tubes. To the last tube add all antibodies in combination.
2. Add 50μl of target cells at 5 - 10 x 10^6/ml, containing 400 μg IgG/ml, to each tube.

3. Incubate cells for 15 min. (45 min for internal antigens), lyse erythrocytes and fix as described in Chapter 6.
4. Acquire data using a flow cytometer.

Data Analysis

1. Display univariate histograms of each file of cells stained with only one antibody as shown in figure 5.
2. Now overlay the univariate histograms for each antibody from the file derived for the antibody combination.
3. The histograms of each single antibody must be identical to that when each is combined. If they are not, there is a problem with the combination or with the instrument set up.

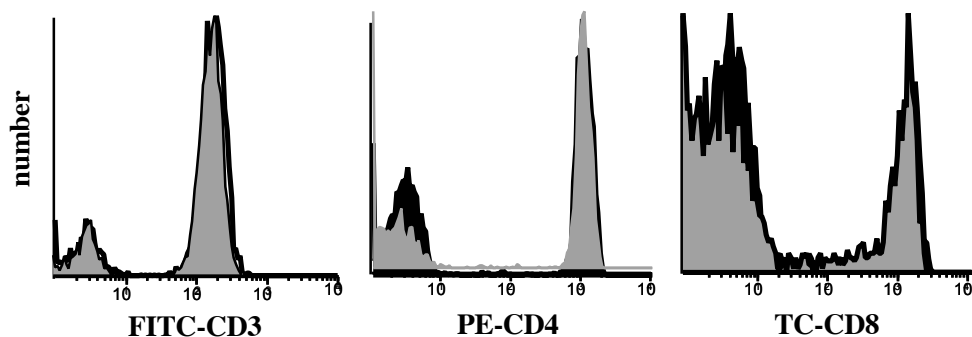**FITC-CD3**   **PE-CD4**   **TC-CD8**

Figure 5. Verification of Antibody Combinations
When producing an antibody combination, the histograms from cells stained with each antibody separately must overlap the histogram from cells stained with the combination, as shown in Figure 5.

Producing a Batch

Once the antibody combination has been validated, batches of the number of tests desired can be created, e.g. 100 test batches. For consistency, to reduce errors and to promote staining efficiency, it is important to create a batch rather than to add the antibodies individually to cells at the time of staining. It is also important that the antibody concentration is three to four times titer so they can be mixed together to give 1x titer because the volume of each antibody dilutes the others.

Materials

target cells for combination to be titered
PBS Phosphate buffered saline without Calcium or Magnesium (Gibco-BRL #21300-082))
normal mouse IgG (Caltag #10400)
12 x 75 mm polypropylene test tubes (Falcon 2052)
ultrapure formaldehyde (Polysciences #04018)
lysing solution (see recipe)
Sorvall centrifuge rotor H1000B or equivalent
previous antibody combination batch
antibody combination batch
isotype control combination
flow cytometer

Procedure

1. To one test tube add the appropriate amount of the antibody combination batch to 50 μl of target cells at 5 - 10 x 10^6/ml, containing 200 μg IgG/ml.
2. To a second test tube add the appropriate amount of the previously prepared batch to 50 μl of target cells at 5 - 10 x 10^6/ml, containing 200 μg IgG/ml.
3. Incubate cells for 15 min. (45 min for internal antigens), lyse erythrocytes and fix, as described in Unit 6.1.
4. Acquire data using a flow cytometer.

Data analysis

1. Display bivariate histograms of FL1 vs FL2, FL3 vs FL2 and FL4 vs FL2, if required, and load file as shown in figure 6, top row.
2. Create a template consisting of regions around discrete target cell populations. Compute the MCF for the two antibodies and record. This will be the standard batch for future comparisons.
3. Load newly prepared batch and compute the MCF values for each antibody, as shown in figure 6, bottom row.
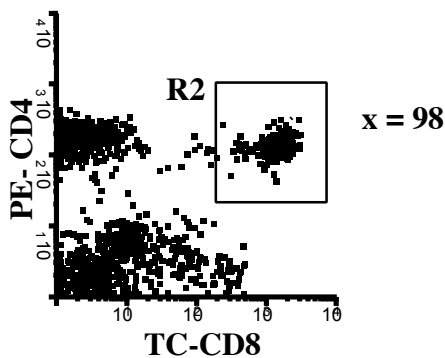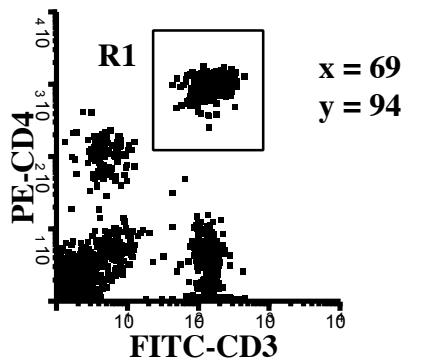
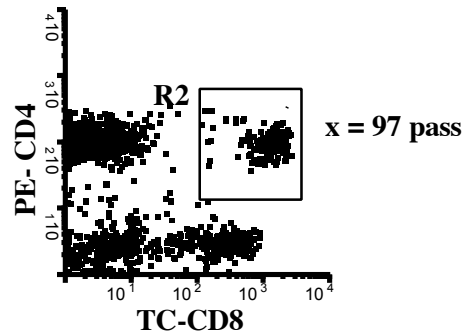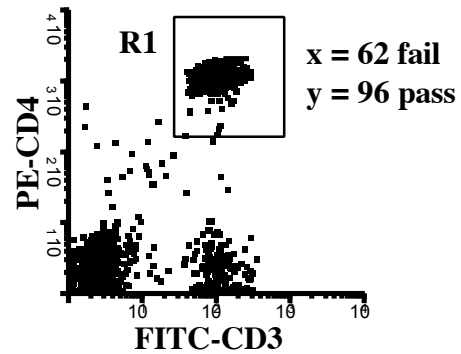**CONTROL BATCH**   **NEW BATCH**

Figure 6. Batch Verification
The newly prepared batch should provide exactly the same pattern as the old batch when the bivariate views of FL1 vs FL2, FL1 vs FL3 and FL4 vs FL2 (if required) are displayed. To quantify the similarity of the patterns, the MCF for selected target cell populations are compared. For a resolution of 128, we accept a +/- 5 channel variation in the MCF for each antibody. Note that the new combination for CD3 fails the test because it is more than 5 channels (69 -62 = 7) from the mean. So the entire batch is not lost, more CD3 would be added and the batch retested. If, however, a newly acquired antibody from a supplier exhibits a difference greater than +/- 5 channel difference, the new MCF value for the antibody is used. (The user has no control over the qc of the supplier.) A variation in MCF greater than +/- 5 channels for a different lot of antibody from the same supplier is not uncommon.

REAGENTS AND SOLUTIONS
Lysing Solution
4.13 g Ammonium Chloride (NH_4Cl) -- Sigma A-5666

0.5 g Potassium Bicarbonate ($KHCO_3$) -- Sigma P-4913

0.0185 g Tetra Sodium EDTA -- Sigma ED4SS
500 ml double distilled water

2% UltraPure Formaldehyde
200 ml Ultrapure, E. M. Grade 10% solution(Polysciences, cat. # 04018)
800 ml Dulbecco's PBS GIBCO (cat#450-1300EC)

BACKGROUND INFORMATION
One of the most important areas of immunophenotyping is using the correct amount of antibody. Most of us depend on the manufacturer to recommend the appropriate amount to use. Unfortunately, this provides a false sense of security because there is no accepted standard for titration. Some suppliers offer a antibodies at a higher concentration while others may actually sell their antibody below titer. One company even offers an antibody at two separate concentrations. Another potential problem occurs when the inappropriate target cell (cell lines, fixed or frozen cells) are used to titer the antibody because the target cell may not be representative of those that will be tested.

It is important to distinguish between specific and non-specific binding. Some Mabs exhibit more non-specific binding than do others. This non-specific binding can easily be determined using the signal to noise (S/N) method for titering. When a "clean" antibody is added at high concentration, there is little change in the position of negative cells and positive cells can be explicitly resolved. Unfortunately, most Mabs bind non-specifically to a lesser or greater extent and this ideal condition is rarely achieved and the amount of non-specific binding increases with antibody concentration producing a "dirty" antibody. Clean Mabs exhibit only epitope positive cells above the marker over a wide range of concentrations, while dirty ones may only exhibit a single optimal concentration. Most Mabs fall between these two extremes.

Because non-specific binding has a much lower affinity than epitope binding it will decrease much more rapidly than specific binding as the concentration of the antibody is decreased. To determine the optional amount of antibody to use it is necessary to determine the greatest amount needed to maximize epitope binding, but not such an excess that non-specific binding

becomes significant. This can easily be done by determining the signal to noise ratio for several antibody dilutions in PBS.

A major reason for the heterogeneity in non-specific binding among Mabs is their structural diversity. Mabs are the product of hybridomas that are created by fusing normal with malignant B-cells. During the screening process, the primary selection criteria is quantity of antibody capable of binding to its epitope and not antibody quality. Indeed, Mabs of the same isotype and subclass are not only structurally different from one another, but they are also different from normal Ig.

In order to properly titer antibodies, it is necessary to have the highest signal (true positive cells) and lowest noise (least number of false positive cells). Fc binding can be blocked using normal mouse IgG (200μg/ml), when biotinylated or directly conjugated antibodies are used. If a second fluorochrome conjugated antibody is used, make sure the blocking IgG is from the same animal species as that of the conjugated antibody.

Antibodies that have proven useful for immunohistochemistry may be inadequate for flow cytometry. This difference is because specific staining represented by bright reaction product can be distinguished from a dimmer general background when observed microscopically. Because the flow cytometer measures total fluorescence, the total difference, rather than the spatial difference, may be so small that no resolution of positive cells occurs.

CRITICAL PARAMETERS
The lysing solution is critical because unlysed red cells may occur in the gated region producing an erroneous denominator. While nearly every supplier of antibodies to human antigens offers a lysing solution of some kind, we recommend using the one described here because of its universal applicability and lack of debris in the suspension. We have tested most of the others and found them to be inferior because they produce variable lysis depending on the source of the specimen and variable amounts of debris. Furthermore, all of them are considerably more expensive than ammonium chloride.

The greatest disadvantage of ammonium chloride is that is must be made fresh daily. This is because NH_4Cl becomes $(NH_4)_2CO_3$ in aqueous solution which has no lysing activity at all. We prepare several individual packets of dried powder and rehydrate the packets as needed just prior to use.

Immunoglobulins spontaneously aggregate and this can be enhanced by freezing and thawing them or by lyophilizing them. Aggregated immunoglobulin may increase nonspecific binding. To remove aggregates, all newly acquired antibodies should be centrifuged at high speed.. This can be performed using an eppendorf type centrifuge or an airfuge. The aggregates can be left in the bottom or the supernatant antibody transferred to a new tube.

IgG at high concentration is used to block Fc receptor binding and to some extent nonspecific binding. Purified mouse IgG can be used in all situations except when the neat antibody is titered. In this instance the blocking IgG must be from the same species as the production

source of second conjugated antibody. This will prevent the second antibody binding to the blocking IgG. For internal antigens it is important to use both epitope positive and negative cell lines. By doing so, the antibodies binding to its specific epitope will be confirmed.

The protein concentration inside cells is orders of magnitude higher than membrane protein thereby exacerbating nonspecific (NSB) binding. Since we desire to resolve specific from NSB binding it is necessary to systematically evaluate both. The isotype control Ig provides an estimate of the NSB component while the antibody is composed of both specific and NSB components. As both are diluted, for acceptable antibodies, the mean channel fluorescence of NSB falls faster then the specific component. The ratio between the MCF of the antibody to isotype must be greater than three to be acceptable.

When concentrations of antibody greater than 3.0 μg/ml are required, the antibody is no good and should not be used, because either the affinity or specific activity is too low. The best antibodies usually stain at concentrations of 0.01 - 1.0 μg/ml. This is equavalent to 10 - 1000ng per test. One should always compute the concentration of antibody at titer. This can be expressed in ng/μl or μg/ml.

An historical record of titers for antibodies can provide information on important formulation changes that maynot be announced by the supplier.

When staining cells for intracellular antigens, an Ig block is usually ineffective for blocking non-specific binding. This is because the binding sink of proteins is so high, that there is no demonstrable effect. When a neat antibody and second fluorochrome conjugated step is combined with other biotinylated, hapten conjugated or directly conjugated antibodies, however, it is necessary to block with IgG.

After the initial 45 min. incubation to allow the antibody or isotype control to enter the permeabilized cells, three ml of PBS is added. The cells are then incubated an additional 45 min. to allow the unbound antibody or isotype control to leave the cells. This extended wash also has the added benefit of reducing NSB more effectively while not affecting specific binding.

It is important to prepare tubes containing the second antibody alone to distinguish between NSB due to Ig, revealed by the isotype control and from that produced by the second antibody itself. If NSB of the second reagent is high, it may be necessary to retiter or find a new supplier. Always use the F(ab)'$_2$ fragment for second antibodies. The unstained cells provide a reference upon which all other binding can be evaluated.

When antibodies are combined for multicolor analysis, it must be determined that they behave independently, i.e. do not interact with one another. Once the combination has been shown to give reliable results, it can then be used for immunophenotyping. It is desirable to make batches of the combination the size of which will depend on the number of specimens to be evaluated before the earliest antibody expires. If an additional batch is required, it needs to be prepared before the current batch has been depleted and its performance verified.

When a new combination is designed it must be tested to verify its intended use. A minimum of 25 normals and 25 patients approximating the ethnic origin and age distribution of the target population needs to be tested. Reference ranges for each resolved population are determined and become a part of the combination's verification record. As the combination is used, newly acquired values can be added to the reference ranges to further enhance the combination's validity.

When combining antibodies each one dilutes the others so that each one is at a different concentration when mixed. Depending on the volumes employed, this can have a profound effect on the combinations staining quality. For example, if three antibodies each have a titer of 100 ng in 20 μl the concentration of antibody after addition to 50 μl of cells is 100 ng/(20 μl + 50 μl) = 1.4 ng/ml, the antibodies 1x titer. If each of the three antibodies has the same titer and we mix them together their combined volume is 60 μl and when the combination is added to 50 μl of cells, the concentration of each antibody is now only 100 ng/ (20 + 20 + 20 + 50) = 0.9 ng/μl. Thus, there is a 36% error for each antibody in the combination's titer and each antibody in the combination is not at the optimal concentration required to stain cells. Thus, when mixing antibodies, the stock concentrations should be high so the volume required is low, to minimize mutual dilution.

For any number of antibodies in combination, the amount of each to mix is determined by simultaneous equations as follows:

$$Ab_1V_1 = C_1(V_c + V_1 + V_2 + ... + V_n)$$
$$Ab_2V_2 = C_2(V_c + V_1 + V_2 + ... + V_n)$$
$$Ab_nV_n = C_n(V_c + V_1 + V_2 + ... + V_n)$$

where Ab_n is the concentration of stock antibody
V_n is the required volume of stock antibody to be combined
C_n is the concentration of Ab_n required to optimally stain cells
V_c is the final volume of the cell suspension after adding all antibodies

As an example, suppose we wish to mix together two antibodies to stain cells in 50μl. We have the following data:

	Ab1	Ab2
stock concentration	10 ng/μl	30ng/μl
titer	1ng/μl	6ng/μl

for Ab1:
$$Ab_1V_1 = C_1(V_c + V_1 + V_2)$$
substituting values and soving for V_1:
$$V_1 = 5.55 + 0.11V_2$$

for Ab2:
$$Ab_2V_2 = C_2(V_c + V_1 + V_2)$$
substituting values and solving for V_2 :
$$V_2 = 11.7\mu l$$

solving for the value of V_1
$$V_1 = 5.55 + 0.11\ V_2 = 6.84\ \mu l$$

Thus, for one test we would mix together 6.84 μl of Ab1 and 11.7 μl of Ab2 and add this to 50 μl of cells for a total volume of 68.54 μl. To make a 100 test batch, we would combine 684 μl of Ab1 with 1170 μl of Ab2 and use 18.5 μl per test for staing cells in 50 μl.

KEY REFERENCE
C.C. Stewart, S.J. Stewart, Cell Preparation for the Identification of Leukocytes. In: Methods In Cell Biology, (Z. Darzynkiewicz, J. Robinson, H. Crissman, eds.) Academic Press, Inc. New York, 41:39-60, 1994.

contributed by Carleton C. Stewart And Sigrid J. Stewart,
Laboratory of Flow Cytometry, Roswell Park Cancer Institute, Buffalo, NY