Titration of Fluorochrome-Conjugated Antibodies for Labeling Cell Surface Markers on Live Cells

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ABSTRACT

Nonspecific antibody binding is best eliminated by optimizing the amount and concentration of the antibody. An antibody titration assay should be applied to determine the antibody amount and concentration resulting in the highest signal of the positive population and the lowest signal of the negative population. While conventional antibody titration protocols focus on the concentration of the antibody, this protocol for antibody titration considers the antibody concentration, as well as the antibody amount. Thus, it is designed to find the optimal antibody concentration for labeling antigens expressed on the surface membrane of live cells, while nonspecific antibody binding is kept to a minimum. *Curr. Protoc. Cytom.* 54:6.29.1-6.29.9. © 2010 by John Wiley & Sons, Inc.

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BASIC PROTOCOL

This procedure is based on the concept that the optimal antibody concentration for live-cell labeling produces the best discrimination between positive and negative cells, while nonspecific antibody binding is kept to a minimum. Accordingly, the most important aim is to keep fluorescence intensity levels of negative cells equal to autofluorescence (i.e., when there is no antibody binding).

The entire procedure consists of two parts. The first part (steps 1 to 24) is designed to determine the maximum amount of antibody that allows for the minimum increase in background fluorescence. The focus of this part is on the fluorescence intensity of the negative cells, rather than the maximum difference between that of positive and negative cells. This maximum amount directly represents the total number of antibody molecules available in the sample for epitope binding. The second part (steps 25 to 40) is designed to determine, with the same amount of antibody, the antibody concentration that produces the best discrimination between positive and negative cells.

Although described and tested for cell surface markers on live cells, it is reasonable to assume that this protocol can be applied to fixed cells, as well as to intracellular antigens.

Materials

Stock solution of fluorochrome-conjugated antibody to be titered
Target cells for antibody titration
Phosphate-buffered saline (PBS; e.g., BioWhittaker, cat. no. 17-516Q) or equivalent

1.2-ml microcentrifuge tubes
2-, 10-, 20-, 100-, 200-, 1000-µl pipets (e.g., Gilson Pipetman)
Pipet tips
Microcentrifuge
12 × 75–mm polystyrene (or polypropylene) test tubes
Flow cytometer

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1. Obtain the concentration of the antibody stock solution from the product label or the product data sheet (frequently available on the manufacturer's Web site). If the antibody concentration has not been supplied with the product, most manufacturers provide the specific antibody concentration when contacted directly (by e-mail or phone).
2. Prepare a cell suspension containing $\sim 2 \times 10^8$ cells/ml.

Determine the maximum amount of antibody to be used

3. Prepare six 1.2-ml microcentrifuge tubes (no. 1 to no. 6) with 5 µl cell suspension ($\sim 10^6$ cells) in each tube.

   NOTE: *At the end of the preparation procedure, each tube will contain 0.5 ml of total assay volume.*

4. Add 0.495 ml of PBS to the first (no. 1) tube (unstained control).
5. Determine the required volume of antibody stock solution for each tube in order to achieve a series of final antibody concentrations [e.g., 10 ng/ml (no. 2); 20 ng/ml (no. 3); 0.1 µg/ml (no. 4); 0.2 µg/ml (no. 5); and 1 µg/ml (no. 6)].

   Please note that this series of dilutions is an example and users might want to choose a different series for their tests. Do not add antibodies until the proper amount of PBS is added to the tubes (step 8).

   For example, if the antibody concentration in the stock solution equals 12.5 µg/ml (12.5 ng/µl), the assay concentration of 10 ng/ml (tube no. 2) is achieved by using 0.4 µl antibody stock solution in a total of 500 µl assay volume (12.5 µg/10 ng = 1250-fold dilution; 500 µl/1250 = 0.4 µl).

6. Calculate the volume of PBS required for each tube that will result in a total assay volume of 0.5 ml.

   For example, X µl PBS = 500 µl total assay volume – (5 µl cell suspension + 4 µl antibody stock solution); then X = 491 µl PBS.

7. Add the calculated volumes of PBS to the respective tubes.
8. Lastly, add the appropriate volumes of the antibody stock solution (calculated in step 5) to each tube and mix gently.

   All steps involving fluorochrome-conjugated products should be performed while products are protected from light exposure. Preferably, light exposure should be <40 Lux (e.g., in a dark laminar flow hood while lights in the laboratory area are kept on).

9. Incubate all tubes for 15 min at room temperature (in the dark by wrapping the tubes with light-protective material or placing them in a laboratory cabinet).
10. Centrifuge for 3 min at 400 × *g*, room temperature.
11. Remove the supernatant by carefully pipetting, gently resuspend the cells in residual fluid by pipetting or vortexing, and then add 1 ml PBS to remove excess antibody.
12. Centrifuge for a second time, 3 min at 400 × *g*, room temperature.
13. Remove the supernatant, carefully resuspend the cells in residual fluid by pipetting or vortexing, and add 1 ml PBS to bring the sample to an appropriate concentration for flow cytometric measurements.
14. Transfer the suspension to polystyrene (or polypropylene) tubes for flow cytometric measurements.
15. Acquire at least 500 events of the positive cell population.

Perform data analysis

16. For each tube, display a bivariate histogram of side light scatter versus forward light scatter.

17. Draw a polygonal region (R1) around the (live) cells, excluding debris.

18. Display the R1-gated events in a bivariate histogram of side light scatter versus fluorescence intensity.

19. For each tube, draw one region (R2) around the negative population and another (R3) around the positive population (Fig. 6.29.1).

20. Determine and record the median fluorescence intensity of the negative population (R1+R2 gated events) and the positive population (R1+R3-gated events) for all six tubes.

    NOTE: *The first tube (unstained control) has a median fluorescence value for the negative population only.*

21. *Optional:* Create a graph in which the median fluorescence values of the negative population (Series 1) and those of the positive population (Series 2) are plotted as a function of the antibody amount (Fig. 6.29.2).

22. Alternatively, use dot (or contour) plots to create a figure as shown in Figure 6.29.3.

    Such visual analysis is especially useful when no discrete positive cell population is present and instead, a continuous "smear" with increasing levels of fluorescence intensity is connected with the negative cell population (e.g., CD38 expression on hematopoietic cells).

23. Calculate the signal-to-noise ratio by dividing the median fluorescence value of the positive cells by that of the negative cells.

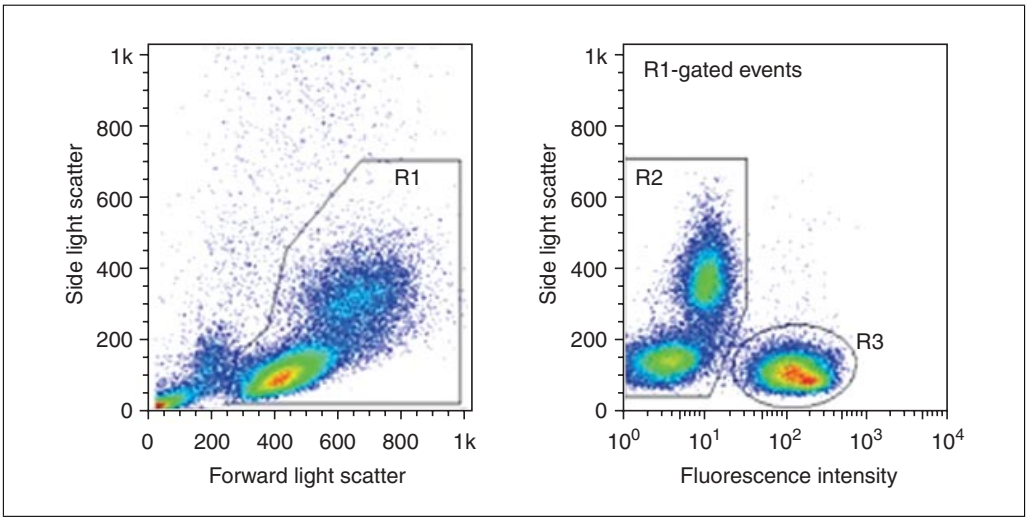

Figure 6.29.1 Typical gating strategy used in data analysis. A side scatter versus forward scatter bivariate histogram (left dot plot) is used to exclude debris by means of a region (R1) drawn around the events that represent cells. Next, only the R1-selected events are displayed in a side scatter versus fluorescence intensity bivariate histogram (right dot plot) and regions are drawn around the negative (R2) and positive (R3) cell populations. Median fluorescence intensities are determined of events gated by R1+R2 (fluorescence intensity of the background) and R1+R3 (fluorescence intensity of the positive cells). For the color version of this figure go to *http://www.currentprotocols.com/protocol/cy0629*.

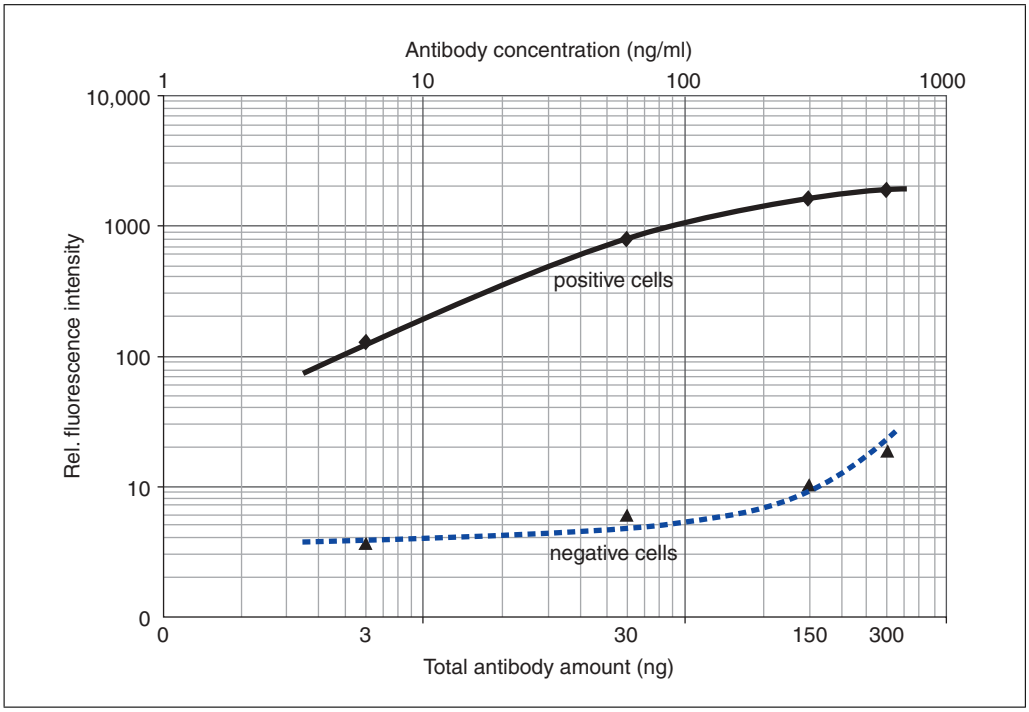

Figure 6.29.2 Determining the maximum amount of antibody. Typical results of the first part (steps 1 to 24) of the Basic Protocol using cells with a distinct positive target population (e.g., CD3-expressing cells). Samples of 500 µl contained 3 ng, 30 ng, 150 ng, and 300 ng of antibody, respectively, and were analyzed as described in steps 16 to 21. Data points reflect median fluorescence values of the positive (series 1) and the negative (series 2) populations. In this example, the maximum amount of antibody that results in distinguishable positive cells while background remains low ranges from 3 ng to 10 ng.

24. Determine the antibody amount where the median fluorescence intensity of the negative population most closely resembles that of the unstained control and the signal-to-noise ratio is high. (The example in Fig. 6.29.3 shows a maximum antibody amount of 5 ng.)

Determine the optimal antibody concentration

25. Prepare five additional microcentrifuge tubes (no. 7 to no. 11) with 5 µl cell suspension ($\sim 10^6$ cells) in each tube.

    NOTE: *At the end of the preparation procedure, each tube will have a different total assay volume.*

26. Calculate the volume of antibody stock solution that is based on the amount of antibody determined in step 24 (e.g., if the amount of antibody determined in step 24 is 5 ng and the antibody stock solution is 12.5 ng/µl, the volume of the antibody stock solution in each tube should be 0.4 µl). Do not add the antibodies until the proper amount of PBS is added to the tubes (step 27). Calculate the remaining volume of PBS required for each tube to result in its respective total assay volume of 1000 µl (no. 7); 500 µl (no. 8); 100 µl (no. 9), 50 µl (no. 10), and 10 µl (no. 11).

    Example, for tube no. 11:

    X µl of PBS = total assay volume of tube no. 11 – (5 µl cell suspension + 0.4 µl antibody stock solution)

    X = 10 – (5 + 0.4) = 4.6 µl PBS.

27. Add the calculated volumes of PBS to the respective tubes.

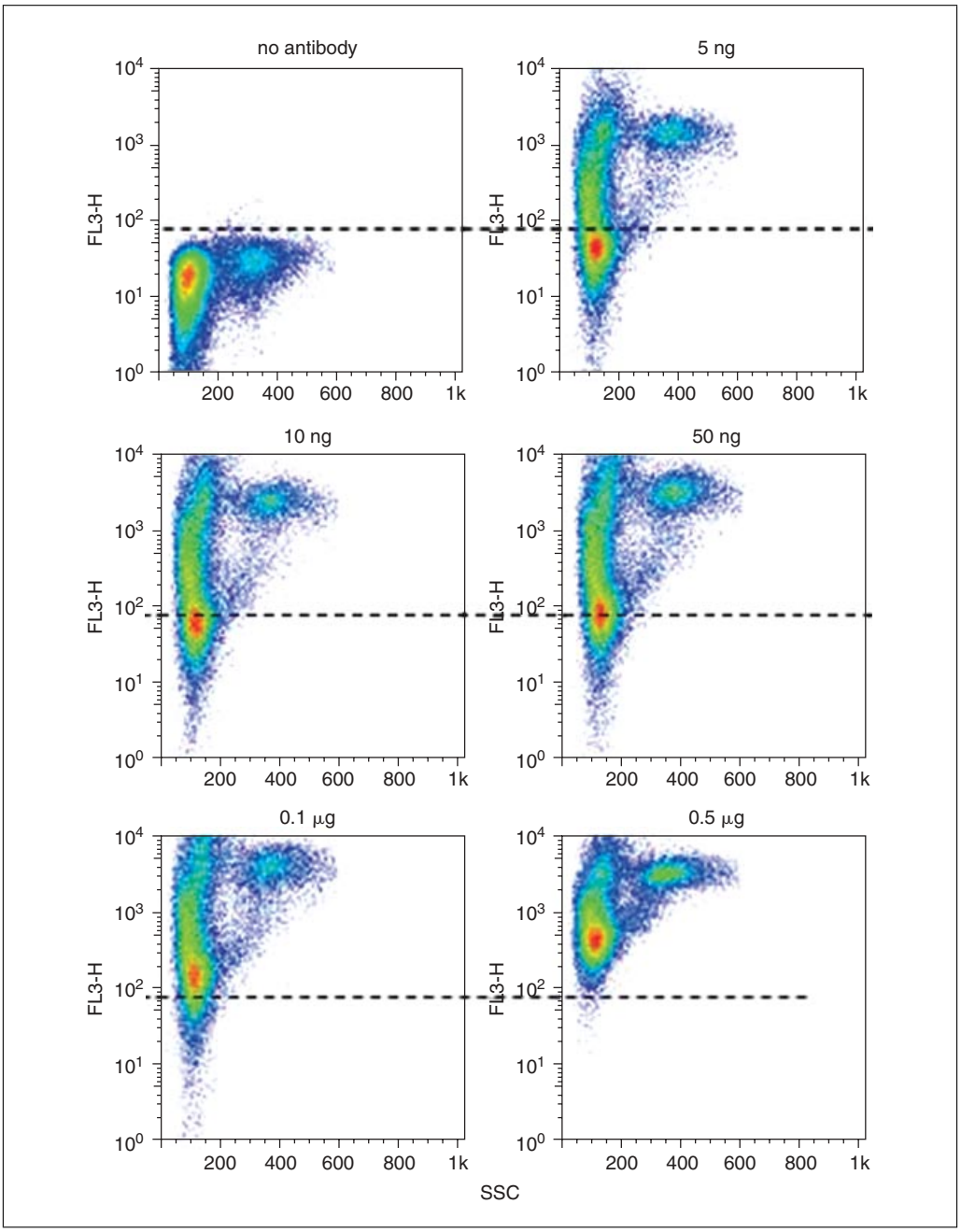

Figure 6.29.3 Determining the maximum amount of antibody. Dot plots of cells with a nondistinct positive target population (e.g., CD38-expressing cells) for visual analysis of results of the first part (steps 16 to 22) of the Basic Protocol. Samples of 500 μl contained 5 ng, 10 ng, 50 ng, 0.1 μg, and 0.5 μg of antibody, respectively, and were analyzed as described in steps 16 to 22. The dashed line indicates the maximum autofluorescence intensity of unstained cells. In this example, the maximum amount of antibody that results in distinguishable positive cells while background remains low is 5 ng. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cy0629*.

28. Lastly, add the appropriate volume of the antibody stock solution, as determined in step 26, to all tubes and mix gently.

29. Incubate all tubes for 15 min at room temperature (in the dark).

30. Adjust the total assay volumes of tubes no. 8 to no. 11 to 1 ml by adding the appropriate amount of PBS (no washing steps are necessary).

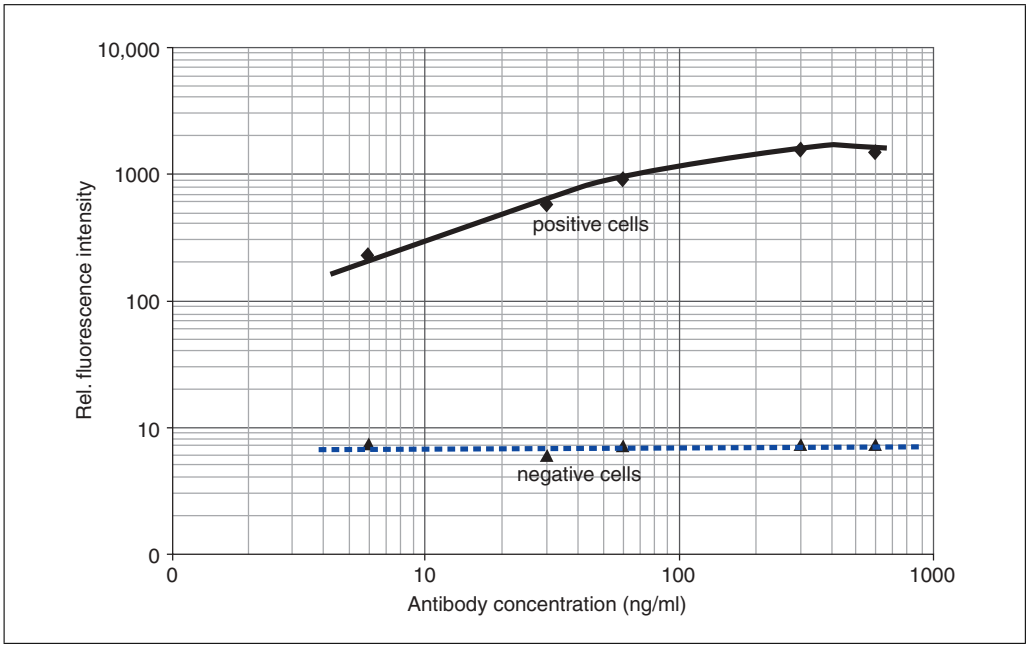

Figure 6.29.4 Determining the optimal antibody concentration. Typical results of the second part (steps 25 to 40) of the Basic Protocol using cells with a distinct positive target population (e.g., CD3-expressing cells). Samples containing 3 ng of antibody were prepared in 500 µl, 100 µl, 50 µl, 10 µl, and 5 µl, resulting in antibody concentrations of 6 ng/ml, 30 ng/ml, 60 ng/ml, 0.3 µg/ml, and 0.6 µg/ml, respectively. In this example, the optimal antibody concentration plateaus at 0.3 µg/ml.

31. Transfer all samples to polystyrene (or polypropylene) tubes for flow cytometric measurements.

32. Acquire at least 500 events of the positive cell population.

Perform data analysis for antibody concentration

33. For each tube, display a bivariate histogram of side light scatter versus forward light scatter.

34. Draw a polygonal region (R1) around the (live) cells, excluding debris.

35. Display the R1-gated events in a bivariate histogram of side light scatter versus fluorescence intensity.

36. For each tube, draw one region (R2) around the negative population and another (R3) around the positive population (Fig. 6.29.1).

37. Determine and record the median fluorescence intensity of the negative population (R1+R2 gated events) and the positive population (R1+R3-gated events) for all six tubes.

38. *Optional:* Create a graph in which the median fluorescence values of the negative population (Series 1) and those of the positive population (Series 2) are plotted as a function of the antibody concentration (Fig. 6.29.4).

    Alternatively, use dot (or contour) plots to create a figure as shown in Figure 6.29.5. Such visual analysis is especially useful when no discrete positive cell population is present, and instead, a continuous "smear" of increasing levels of fluorescence intensity is connected with the negative cell population (e.g., CD38 expression on hematopoietic cells).

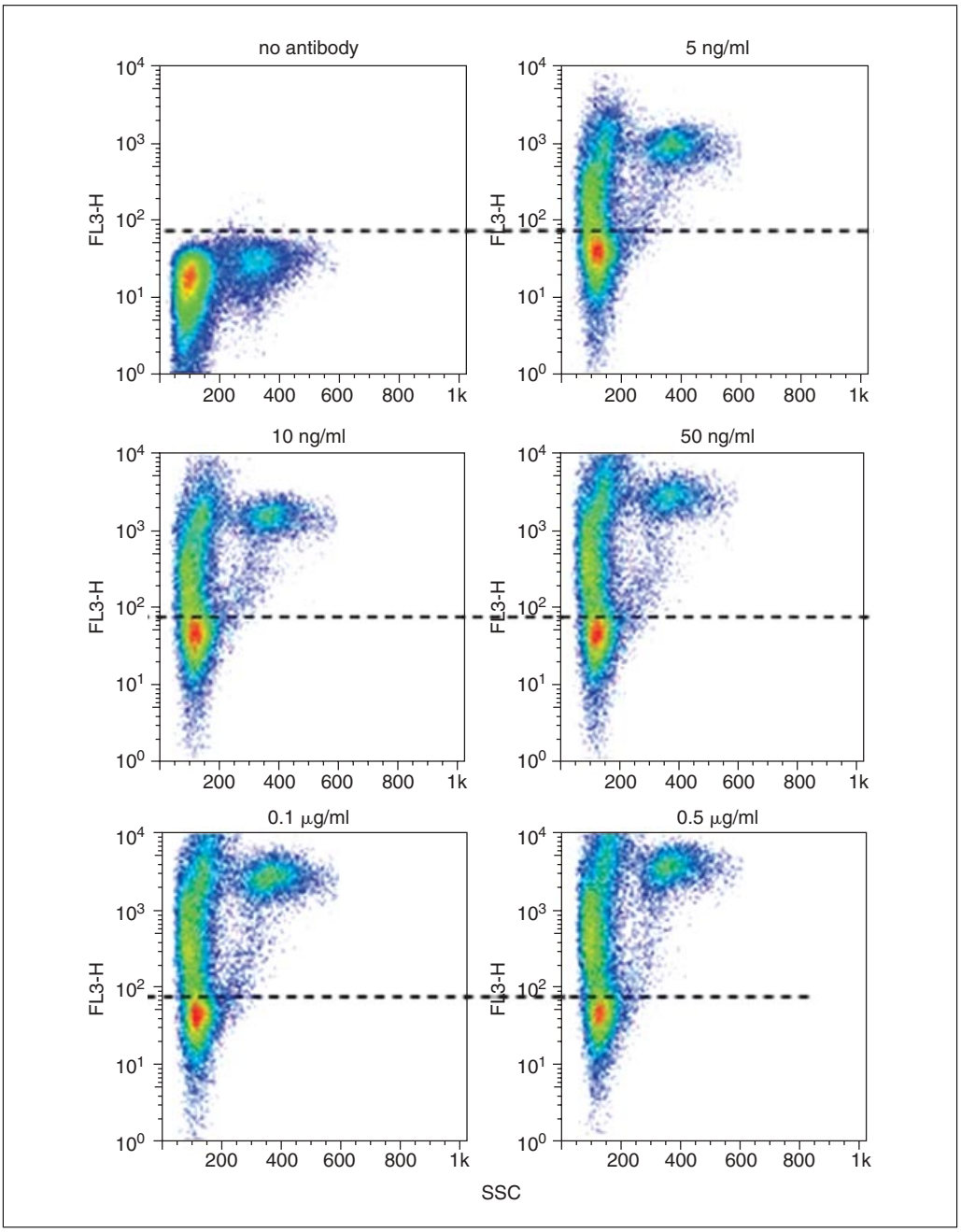

Figure 6.29.5 Determining the optimal antibody concentration. Dot plots of cells with a nondistinct positive target population (e.g., CD38-expressing cells) for visual analysis of results of the second part (steps 25 to 40) of the Basic Protocol. Samples containing 5 ng of antibody were prepared in 1000 μl, 500 μl, 100 μl, 50 μl, and 10 μl, resulting in antibody concentrations of 5 ng/ml, 10 ng/ml, 50 ng/ml, 0.1 μg/ml, and 0.5 μg/ml, respectively. The dashed line indicates the maximum autofluorescence intensity of unstained cells. In this example, the optimum concentration may be even higher than 0.5 μg/ml. For the color version of this figure go to *http://www.currentprotocols.com/protocol/cy0629*.

39. Calculate the signal-to-noise ratio by dividing the median fluorescence value of the positive cells by that of the negative cells.

40. Determine the antibody concentration where the signal-to-noise ratio is highest. (The optimum antibody concentration in the example shown in Fig. 6.29.5 is 0.5 μg/ml).

COMMENTARY

Background Information

A conventional antibody titration protocol varies the total amount of antibody (i.e., the absolute number of antibody molecules) in a set assay volume (Kantor and Roederer, 1996; Owens and Loken, 1997; also see *UNIT 4.1*). Consequently, the result of increasing antibody concentrations also reflects the result of increasing the total number of antibody molecules. Because the expected negative cells do not express the antigen of interest, an increase in fluorescence intensity of the negative cells is generally due to nonspecific antibody binding. When nonspecific binding occurs on negative cells, it is possible that it occurs on the positive cells as well (in addition to the expected specific binding). Nonspecific antibody binding is expected to occur when the total number of antibody molecules greatly exceeds the number of target antigens. Test samples with high antibody concentrations prepared according to conventional antibody titration protocols also contain high antibody amounts and are therefore highly susceptible to nonspecific binding (Srivastava et al., 1992; Hulspas et al., 2009; also see *UNIT 4.1*). Consequently, the conventional antibody titration protocol may not result in an optimum antibody concentration at which the negative cells display the lowest signal, thus underestimating the true optimum antibody concentration.

Although the first part (steps 1 to 24) of this antibody titration protocol is essentially identical to conventional titration protocols, the interpretation of the results is very different. Rather than the optimum antibody concentration, the results are used to determine the maximum antibody amount (i.e., number of antibody molecules) that can bind to a certain number of antigens without a significant degree of nonspecific binding.

The maximum antibody amount determined in steps 1 to 24 is based on the number of available epitopes present on the target cells. Although it is generally assumed that more antibody molecules are needed when more epitopes are available (Kantor and Roederer, 1996), a simple epitope titration (in which the same amount of antibody molecules are used to label an increasing number of target cells) is likely to show that the antibody amount, as determined in steps 1 to 24, is able to label over 50-fold more target cells without significant signal reduction (as long as cells and antibody are kept at high concentrations during labeling).

Critical Parameters and Troubleshooting

The series of antibody dilutions (10, 20, 100, 200, 1000 μg/ml) described in step 5 deviate from the standard 2-fold dilution series (Kantor and Roederer, 1996) in order to limit the number of test samples while covering a wide range of conditions. Depending on the reagent, other antibody dilution series may be used. During the preparation of the samples, it is essential that the antibody be added last. This is to ensure that the labeling occurs under each intended kinetic condition. Because time plays an important role in kinetic processes, antibody incubation times used in this protocol should also be used in the actual experiment for which the antibody amount and concentration needed to be determined.

Titrated antibodies for which the positive populations appear as a continuous smear of increasing levels of intensity connected to the negative population, make it difficult to determine the median fluorescence intensity. In these cases, visual analysis (as shown in Figs. 6.29.3 and 6.29.5) can be used to determine the optimal cell labeling conditions.

When working with live cells, it may be difficult to accurately set the first region (i.e., excluding debris) due to a high content of dead cells. In that case, the analysis can be improved significantly by adding a dead cell indicator (e.g., propidium iodide or 7-amino-actinomycin D) prior to flow cytometric measurements. Accordingly, a bivariate histogram of this dye versus forward (or side) light scatter should be created and a region (R0) set around the live cells for further analysis.

Determining the optimum antibody concentration, as described in steps 25 to 40 of this protocol, is limited by the antibody concentration in the stock solution. Obviously, it is not possible to prepare test samples with higher antibody concentrations than formulated in the stock solution. This may be particularly relevant for low-affinity antibodies where optimal antibody concentrations for cell labeling can be very high, while the amount of antibody should be kept very low in order to minimize nonspecific binding. In such cases, it may be possible to use 10- to 50-fold more target cells (by working from a starting total cell concentration of 2×10^9 to 1×10^{10} cells/ml) to make more epitopes available to the antibody molecules.

When the cell sample contains Fc-receptor-expressing cells (e.g., NK cells, macrophages, mast cells, neutrophils), the antibody being titrated could also act as a ligand and bind to Fc-receptors. Hence, blocking of Fc-receptors prior to the antibody titration procedure is important. Proper blocking of Fc-receptors can be verified with a matching isotype control antibody.

The titration protocol relies entirely on the presence of a negative cell population. However, there are cases (e.g., studies with cell lines or commonly expressed antigens) in which the cell sample does not contain a negative population. Here, an acceptable way to establish the proper antibody amount and concentration is to create a mixture of cell types by adding a small number of other (but very similar) cells that are known to lack the antigen of interest to the original cells.

Anticipated Results

If the antibody concentration of the stock solution is sufficiently high, it is possible to reach a plateau value for the fluorescence intensity of the positive cells while the negative cells' intensity stay as low as that of autofluorescence (as shown in Fig. 6.29.4). A high antibody concentration of the stock solution allows for testing in very small assay volumes. Typically, the highest antibody concentration on a high cell concentration using a low amount of antibody, results in the highest signal of the positive population and the lowest signal of the negative population.

Time Considerations

The entire titration procedure should take no more than 4 hr, including data analysis. Time required to prepare the target cell population can vary significantly and should be taken into consideration. Furthermore, the flow cytometer should be set up and ready for measurements prior to the sample incubation to ensure efficiency.

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