BD™ Digital Flow Cytometers

Establishing Optimum Baseline PMT Gains to Maximize Resolution on BD Biosciences Digital Flow Cytometers

Specific Aims

This application note describes a procedure for optimizing cytometer settings for digital pulse area measurements by establishing baseline detection levels using a dim calibration particle or bead. These optimized settings establish an overall detection level where unstained and brightly stained cells are properly displayed on scale with minimal interference from electronic noise. In addition, when this procedure is used for instrument setup, it results in maximum instrument resolution. Since a mid-intensity calibration particle can then be run on subsequent days as verification of the optimized settings, this procedure simplifies monitoring and tracking of day-to-day performance (instrument quality control).

The method described here can be applied to any BD Biosciences digital flow cytometer to measure pulse area (total fluorescence); for this application note we used a BD™ LSR II flow cytometer and BD FACSDiva™ software. Optimal instrument settings must be established for each specific set of optical filters used on the instrument platform. Minor adjustments can be made to fine-tune settings for experimental samples, and these optimized settings can be saved as application-specific detector settings for all experiments of the same type. With minor changes, this method is also applicable to the BD FACSCanto™, the BD FACSVantage™ SE digital option, and the BD FACSAria™ flow cytometers using BD FACSDiva software.

Introduction

Visually establishing photomultiplier tube (PMT) voltages, or instrument gain, using unstained cells or other methods, is not the best approach for setting up the new multi-laser, multicolor digital flow cytometers currently available. It is possible to adequately set up single-laser systems using 488 nm excitation and collecting fluorescence in the fluorescein (FITC) and R-phycoerythrin (PE) channels based on cellular autofluorescence, since the detection of autofluorescence is easy in the FITC and PE channels. However, determining appropriate gain when using probes such as APC and the tandem fluorophore APC-Cy7 is problematic, because cellular autofluorescence is negligible in the longer near-infrared wavelengths. For multi-laser, multi-color systems, the long-held rules for optimizing PMT gains visually no longer apply.

Introduction *(continued)*

The optimum setup method relies on a positive signal detected in each channel of interest.[1] The method must ensure that the instrument gain for each detector places the unstained populations above electronic noise, to optimally discriminate dim populations from background, while maintaining high resolution. Assuring optimal discrimination of real signal from background must be approached differently when using digital electronics in order to make accurate measurements in multicolor flow cytometry.

Most flow cytometry laboratories use empirical methods and materials to set up and standardize their flow cytometry instruments. To be effective, these methods have to account for a particle's velocity through the laser beam, the type of fluorophores in use, bandpass or dichroic filter changes, selection of laser lines and power, and instrument optical drift over time. Clearly, optimally setting up a flow cytometer for complex multicolor flow cytometry measurements requires more than reliance on just visual cues.

The following method is based on the physics of fluorescence signal generation and detection.[1, 2, 3, 4] Baseline settings are established using pulse area for a given flow cytometer by measuring the percentage of the coefficient of variation (%CV) across a serial range of detection voltages using dim fluorescent particles. It takes into account key variables that can lead to general instrument measurement errors, including the excitation wavelength (determined by the laser), the emission collection band (filters in the optical path), and the characteristics of the detectors and detection electronics (amount of inherent electronic noise). In addition, instrument settings may be refined for each application, further minimizing potential measurement variation. If any of the key factors (lasers or filters) are changed, the optimal baseline settings must be re-established with the new variable.

Instrument Overview

- Flow cytometer: The examples in this application note are from a BD™ LSR II flow cytometer operating with BD FACSDiva™ software v4.1 using a two-laser, 6-color configuration as shown in *Table 1*.

  Note: It is generally applicable to the Red and Yellow channels of the BD FACSArray™ bioanalyzer, which use PMTs for detectors, but not to the Far Red and NIR channels, which use avalanche photodiodes (APDs) and require a different setup procedure.

Table 1. BD™ LSR II instrument configuration for this application note

FLUOROPHORE (PARAMETER)	LASER	BANDPASS FILTER	DETECTOR LOCATION
FITC	488 nm (Blue)	530/30 nm	Octagon E
PE	Blue	585/42 nm	Octagon D
PerCP-Cy5.5	Blue	695/40 nm	Octagon B
PE-Cy7	Blue	780/60 nm	Octagon A
APC	635 nm (Red)	660/20 nm	Red Trigon B
APC-Cy7	Red	780/60 nm	Red Trigon A

Materials

MANUFACTURER	MATERIAL	CAT. NO.
BD Biosciences	Rainbow Fluorescent Particles, 3.0-3.4 mm (Mid-range calibration particles)	556298
Spherotech, Inc.	Rainbow Calibration Particles, 3.0-3.4 mm, Peak 2 (Dim calibration particles)	RCP-30-5A-2
BD Biosciences	BD FACSFlow sheath fluid	342003
BD Biosciences	BD Falcon round bottom tubes (12 x 75 mm), 5 mL	352008

- Additional software: Microsoft® Excel or similar spreadsheet application

Instrument Optimization Overview

Perform this procedure after the normal instrument startup recommended in the instrument user's guide.

The following is an overview of the instrument optimization process.

- Turn on the instrument
- Check configuration and optics
- Check WE, LD, and ASF*
- Create or open Experiment template
- Acquire DIM beads at various PMT voltages
- Analyze data and enter PMT voltages
- Establish target channels with MID beads

  *WE, LD, and ASF refer to window extension, laser delay, and area scaling settings for the instrument and are optimized according to the procedures outlined in the instrument user's guide.

Preparing the Instrument

To prevent high %CV values caused by unwanted ambient light, install all of the instrument's light shields.

1. Warm up the instrument and lasers for a minimum of 1 hour before beginning this procedure.

2. Perform routine instrument startup and QC to establish appropriate threshold, laser delay, area scaling factor, and minimum window extension settings. Refer to the instrument user's guide.

   Note: On the BD FACSAria™, verify ASF for forward scatter and on fluorescent parameters for each laser.

   Note: On the BD FACSCanto™, window extension should be left at the default setting of 7.00. Changing this could result in an inaccurate laser delay setting.

Method

While performing this procedure:

- Complete all acquisition steps without interruption.

- Keep compensation turned off during acquisition or ensure all compensation values are at zero.

- Ensure the scatter gate for defining the beads is as tight as possible and excludes aggregates of the particles of interest or other interfering particles.

Preparing Calibration Particles

Dim calibration particles are used to establish instrument performance curves based on a range of PMT voltages. Mid-range calibration particles are used to create target channels for the instrument.

1. Label two 12 × 75-mm tubes "DIM" and "MID."

2. Dispense 1 mL of BD FACSFlow™ sheath fluid into each tube.

3. Gently vortex the dim calibration particles and add 2 – 3 drops to the corresponding tube; repeat for the mid-range particles.

4. Vortex the diluted bead tubes and set aside.

   Note: For optimal results, the beads should be used within 8 hours after being diluted.

Creating the Experiment Template

This section describes how to set up a BD FACSDiva™ Experiment template with tube-specific voltage settings for generating instrument performance curves. Create a series of tubes in the Browser that spans the instrument settings range of 300 volts to 900 volts at 50-volt increments. Establish the scatter settings for the first tube and then create the remaining 12 tubes. This will allow all 13 data files to be quickly recorded.

   Note: Some features in this procedure are available only in software version 4.1 or later.

1. Launch BD FACSDiva software.

2. Log into the software.

3. Verify the current instrument configuration as follows:

   a. From the main menu, choose Instrument > Instrument Configuration.

   b. Ensure that the Current Configuration chosen in the software matches the parameters, laser, and detectors on the instrument.

   c. Confirm the instrument contains appropriate filters in the corresponding octagon and trigon slots.

   For this example, the configuration contains the parameters shown in Table 1.

   Note: If the appropriate filters are not installed, replace them with the correct filter(s) for the fluorochromes being measured. Refer to the instrument's user's guide for more information.

4. Create a new data folder in the Browser and name it Baseline Settings.

5. Create a new Experiment in the Baseline Settings folder and name it *Settings mmddyy* (where *mmddyy* is today's date).

6. In the Experiment Inspector, deselect the checkbox *Use global instrument settings*.

This ensures that each Tube's settings will remain fixed as you change instrument settings for subsequent Tubes.

Uncheck
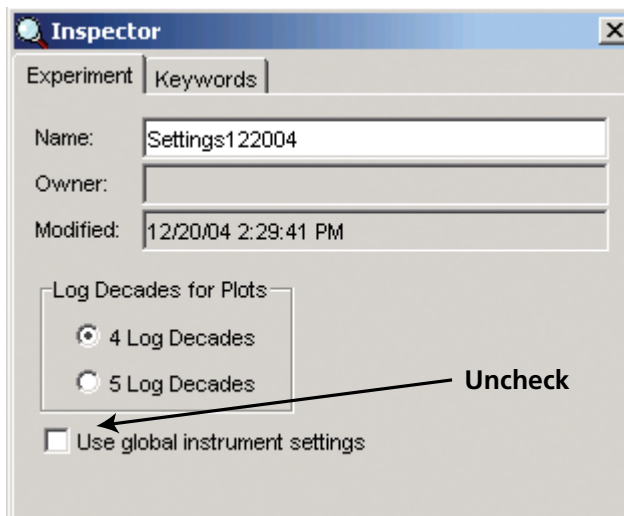
7. Add a Specimen to the Experiment.

8. Add Instrument Settings under the Tube. This is done by selecting the Tube, and then going to the Browser toolbar and clicking on the instrument settings icon.

9. In the Acquisition Controls frame, set the Events to Record to 10,000 for the Tube.

10. Create a FSC-A versus SSC-A dot plot on the global worksheet. For BD FACSCanto™ instruments, create a FSC-H vs SSC-A plot.

    Note: It is not necessary to create individual histograms or bivariate plots of the fluorescence parameters only.

Dot plot

11. Install the DIM bead sample tube on the cytometer.

12. Click the Current Tube pointer next to the Tube in the Browser.

13. Click once on the pointer to start acquisition.

    The pointer turns yellow, indicating acquisition is in process. Events appear in the scatter plot.

14. Ensure the flow rate is within 200 – 500 events per second.

    Note: If the event rate is not within this range, adjust the bead concentration by adding either more beads to the tube or more sheath fluid.

15. Adjust the FSC and SSC voltage settings to position the main bead population at approximately 80,000 on each axis (*Figure 1*).

16. Draw a tight P1 gate around the bead cluster (*Figure 1B*).

    Note: The scatter gate for defining the beads must be as tight as possible to exclude aggregates of the particles of interest or other interfering particles. Small numbers of aggregates or other contaminating particles can significantly reduce the accuracy and usefulness of the measurements.
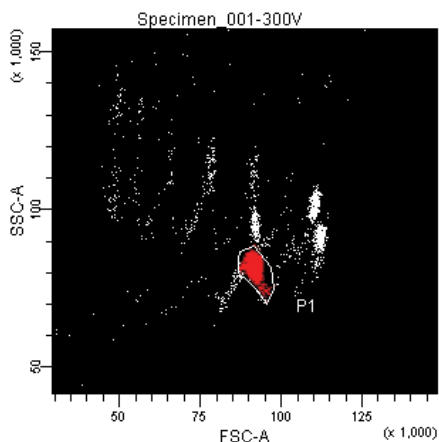
1A
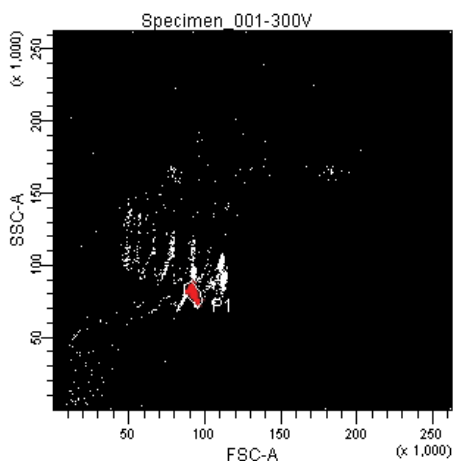
1B

Figure 1. Profile of dim calibration particles (1A) in a zoomed-in plot (1B).

17. Click once on the current tube pointer to stop acquisition and remove the bead tube from the cytometer.

18. Click Next in the Acquisition Controls 12 times for a total of 13 tubes.

 Each Tube will be used to collect data for a fixed PMT voltage setting ranging from 300 volts to 900 volts. Thirteen data points are collected to determine an accurate baseline setting for each parameter.

19. Name the first Tube *300*, the second *350*, the third *400*, and so on. The last Tube should be named *900*.

20. Set the Current Tube pointer to the 300 Tube. In the Instrument frame, set all of the fluorescence parameters to Log and set all voltages to 300 volts. Do not change FSC and SSC.

 Note: Make sure you move the Current Tube pointer to the appropriate Tube *before* you change the voltages.

21. Repeat step 20 for all remaining tubes, using the name of the tube as the voltage setting for all PMT instrument settings for that tube.

22. Click Save in the Workspace toolbar to save your work.

23. Export the Experiment as an Experiment template.

Exporting the Experiment as a template before the data is recorded will allow it to be used again at a later date. For assistance, refer to the *BD FACSDiva™ Software Reference Manual* or online help.

Recording Data

Using the Baseline Settings Experiment template you just created, data will be recorded for the dim calibration particles at the specified voltage settings.

 Note: Do not stop in the middle of performing this procedure.

1. From the General tab, go to Edit > User Preference, and select *Start Acquisition on Pointer Change*.

2. Install the DIM bead sample tube on the cytometer.

3. Click the Current Tube pointer next to the 300 Tube in the Browser.

 The pointer turns yellow, indicating acquisition is in process. Events appear in the scatter plot.
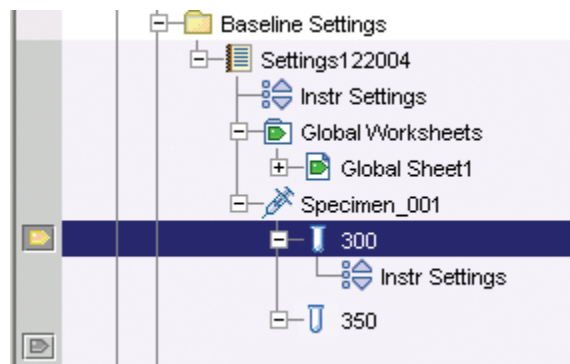
4. Click Record.

5. Click Next and click Record.

6. Repeat step 5 for the remaining 12 Tubes in the series.

 Note: It is important to collect the data sequentially using the same sample.

7. After you have recorded data for all 13 Tubes, deselect the *Start Acquisition on Pointer Change* preference.

Determining the Optimal Baseline Instrument Settings

The batch analysis feature is used to generate an exported statistics file containing results from all recorded Tubes. The resulting %CV and voltage data is then used to create the instrument response curves.

1. Create a statistics view and edit the view to show only the %CV for each fluorescence parameter for the P1 population.

2. Use the Batch Analysis feature to generate a statistics file for the Experiment:

   a. Right-click Specimen and choose Batch Analysis.

   b. In the Batch Analysis dialog box, set the view time to zero, and select the checkbox to export statistics. (It is not necessary to print results.)

   c. Click Start.

4. When the batch is finished, open the resulting statistics file in Microsoft Excel (*Figure 2*).

   Notice the Tube Name column lists the voltage settings, with parameter CVs listed in adjacent columns. (The title rows at the top of the spreadsheet were added.)


Figure 2. Microsoft Excel spreadsheet showing exported data

5. Use the Microsoft Excel Chart Wizard ( ) to create an XY (scatter) graph with data points connected by lines.
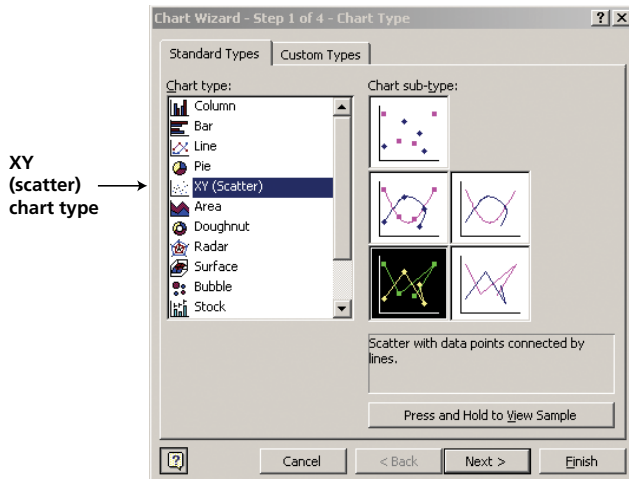
6. Follow the steps in the Chart Wizard to generate the graph:

   • In step 2, select the data range on the worksheet.

   • In step 3, enter PMT Voltage for the title of the x-axis and %CV for the y-axis, and click Finish.

7. In the resulting graph, right-click the y-axis and choose Format Axis. In the Scale tab view, select the Logarithmic scale checkbox, and change the minimum value to 10 (see the following figure). Accept all other default settings. Click OK.
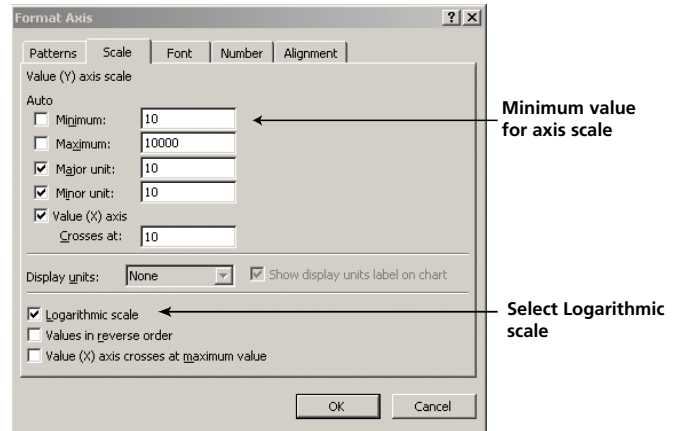
8. Similarly, edit the minimum value for the x-axis scale to 200. The x-axis scale should remain linear. Ignore warnings regarding negative values on logarithmic plots.

   An example of the plotted data is shown in *Figure 3*.

9. Determine the inflection point for each performance curve as shown in *Figure 3*.

The inflection point is the point at which the %CV begins to level off (the first point in which the slope begins to flatten). The corresponding voltage is the minimum recommended voltage for that fluorescence detector.
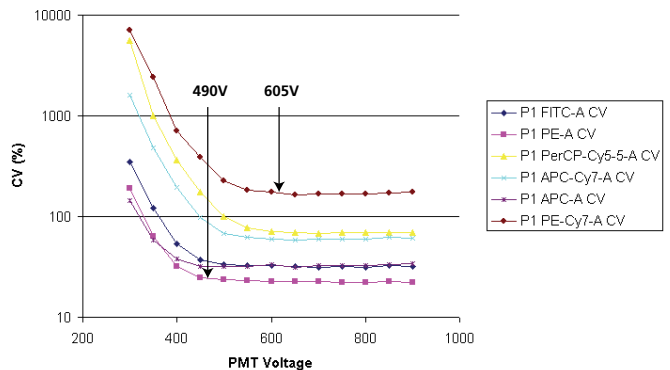

Figure 3. Analyzing the instrument response curves and determining the voltage required for optimal resolution sensitivity.

Creating a Daily QC Template Using Baseline Voltages

Once the baseline instrument voltages are established, a standard calibration particle, such as the BD Biosciences mid-range calibration particle, is used to determine target channels for each detector. Use these target channels to perform simple and routine checks on the system without having to run the dim bead procedure again.

> *Note*: When the optical components of the cytometer are changed—lasers, filters, or mirrors—you need to establish new baseline settings to account for the new elements.

1. Create a new global worksheet, and add a Specimen and Tube to your Experiment.

2. On the new global worksheet, create a FSC-A vs SSC-A dot plot, and fluorescence histograms for each parameter in the Experiment (all the parameters that you will be tracking on a daily basis).

3. Create and set up a statistics view showing the %Parent, %CV, and the mean fluorescence value for each detector.

4. Input the optimal baseline voltages that were determined from the previous section as the voltage settings for the Tube.

5. Record data for the mid-range calibration particles at these optimal baseline voltages.

6. Draw a tight gate around the singlet bead events in the FSC vs SSC plot.

> **Tip**: Use the Zoom feature to magnify the area of interest.

7. Set the fluorescence histograms to display data only from P1. Draw an Interval gate around each fluorescence-positive peak as shown in *Figure 4*.

Ensure that the left and right boundaries define 99% of the bead distribution.

> Tip: Use the %Parent value in the statistics view to verify the gate boundaries.
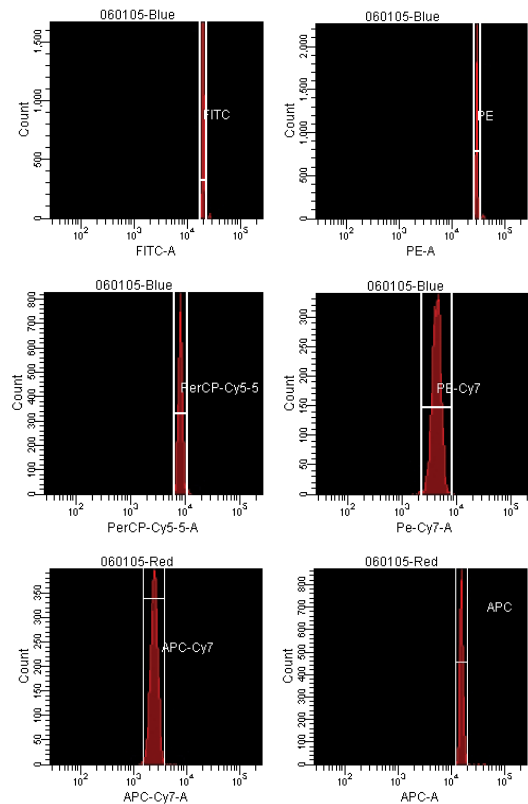

Figure 4. Histograms showing mid-range calibration particles at optimal baseline voltages. The interval gates in each histogram will determine the target channel value for each parameter.

8. From the mean fluorescence value for each detector, determine the target channel using the statistics window for the mid-range calibration particles as shown in *Table 2*.

Run the mid-range calibration particles each day to check instrument performance and make sure the beads are falling into the target channel values for the determined baseline voltages. Determine the acceptable range for your laboratory.

> Tip: These mid-range calibration particle target values should be the starting place for each new experiment. Post this information on the calibrated instrument for each investigator's use.

DETECTOR	BASELINE VOLTAGE	TARGET CHANNEL*
FITC	550	22,400
PE	560	32,400
PerCP-Cy5.5	640	12,600
PE-Cy7	650	3,500
APC	600	24,300
APC-Cy7	670	3,200

*Target channel values rounded to the nearest 100.

Table 2. Example of minimum baseline voltages and established target channels for mid-range calibration particles (on the BD LSR II)

9. Export the Experiment or the analysis as a template.

   To include instrument settings, export an Experiment template. If you plan to do instrument QC within the same Experiment every day, export only the analysis. Once the template is saved, it can be copied and used for daily instrument QC.

10. During daily instrument QC, run the mid-range calibration particles using the saved template and record the channel values obtained for each detector/voltage and compare it to the previous day's results.

> **Tip:** A Levey-Jennings plot for daily channel values is recommended to track trends. For more information on these plots, visit www.westgard.com/levjens.htm

Summary

This rapid and straightforward method of establishing baseline instrument detector voltages can be applied to any of BD Biosciences digital flow cytometers. The overall performance of the cytometer becomes predictable over a wide range of experiment conditions, resulting in day-to-day consistency. This method uses inexpensive materials and is comprehensive enough to account for different excitations and emission configurations for a variety of instruments. The resulting instrument voltages are well suited for detecting and resolving each fluorophore optimally and reproducibility.

The minimum established voltages should be regarded as a starting point for experiment setup. You can fine-tune settings under certain conditions, as covered in the second application note in this series.

Our experience using this method indicates that in situations where the positive sample is off scale (extremely bright), it is appropriate to reduce the detector voltage to bring the fluorescence signal on scale. However, more judgment is required when the background level of fluorescence on a particular sample is approaching mid-scale, as is sometimes the case for intracellular staining. The tendency is to lower the detector voltage back into the first decade. This is permissible only if the decreased settings neither negatively impact compensation nor diminish the ability to distinguish the negative events from background noise.

Using these baseline instrument settings as a starting point, you can determine and save experiment- or application-specific settings.

> **Tip:** Mid-range calibration particles are also useful for establishing experiment-specific target channels. Briefly, you first analyze experiment control samples using the baseline settings just established. The population of interest should be well defined using scatter gains to ensure gating accuracy. You then check the positive population peak for each parameter to ensure the signal is on scale. If it is not, lower the voltage for that detector. Once experiment control settings are established, analyze the mid-intensity particle again and save the new target channel settings for this experiment type. This topic is also discussed in more detail in the second application note.

References

1. Kuhlmann CW, Trotter J. *Setting fluorescence gains for optimum signal to background.* Abstract #0695. Presented at ISAC XXII, Montpellier, France, May 2004.

2. Wood JCS, Hoffman RA. Evaluating fluorescence sensitivity on flow cytometers: an overview. *Cytometry.* 1998;33:256-259.

3. Chase E, Hoffman RA. Resolution of dimly fluorescent particles: a practical measure of fluorescence sensitivity. *Cytometry.* 1998;33:267-279.

4. Hoffman, RA, Kuhlmann, CW. *Practical measures of fluorescence sensitivity demonstrated with the BD FACSArray Bioanalyzer.* Abstract #0691. Presented at ISAC XXII; Montpellier, France, May 2004.

Resource

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