

Standardizing Application Setup Across Multiple Flow Cytometers Using BD FACSDiva™ Version 6 Software

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Technical Bulletin

Contents

1	Abstract
2	Introduction and Background
4	Part 1. Characterization of a Cytometer
6	Part 2. Rules Used to Create Application Settings
7	Part 3. Creating the Application Settings
8	Part 4. Cytometer Standardization
11	Part 5. Verification of Standardization Accuracy
12	Appendix
16	Acknowledgments

Abstract

Standardizing application setup can ensure consistency of results over time across multiple systems. To achieve this consistency, each cytometer must be properly and efficiently characterized and its performance levels maintained. BD™ Cytometer Setup and Tracking (CS&T) software and BD™ Cytometer Setup and Tracking beads (dim, mid, and bright) were developed to deliver this characterization and performance maintenance capability.

In this technical bulletin, we describe how users can create and save the optimal settings for specific applications (called *application settings* in BD FACSDiva™ software) using BD CS&T bright bead target values for each fluorescence detector, to obtain consistent and reproducible results over time. As needed, these CS&T bright bead target values can be used to reproduce application settings on other cytometers with identical optical configurations, or to create new application settings. Multiple cytometers with different optical components such as lasers, laser power, and filters also can be standardized. This technical bulletin explains the principles underlying application settings and the standardization process, and provides details on how to:

- Create optimal application settings
- Save the settings using CS&T bright bead target values
- Standardize multiple cytometers

Introduction and Background

Before standardizing cytometers and creating application settings, users should review the following key concepts described in this section: how the BD Cytometer Setup and Tracking system works, considerations when using highly autofluorescent cells, and the dynamic range of fluorescence intensity. These concepts will enable users to follow the detailed protocols at the end of this technical bulletin to establish optimized, consistent, and reproducible cytometer settings for immunofluorescence applications.

BD Cytometer Setup and Tracking System (CS&T)

With the advent of fully digital flow cytometers, all measurement data is converted directly from instrument detectors, and whenever viewed over several decades of dynamic range, the data is scaled in software rather than with logarithmic amplifiers during acquisition. This approach provides the ability to more precisely characterize and track cytometer performance automatically, on a routine basis. The BD CS&T system uses digital data to accurately characterize the flow cytometer and then ensures reproducible performance day to day. Five key performance metrics are measured for each of the cytometer's detectors for both characterization and tracking:

1. Robust standard deviation of electronic noise (rSD_{EN})
2. Relative detection efficiency for each detector (Qr)
3. Relative optical background (Br)
4. Robust coefficient of variation for each setup bead (rCV)
5. Linearity maximum channel (the maximum acceptable signal level for good linearity)¹

See the Technical Note *Robust Statistics in BD FACSDiva 6.0 Software* (23-9609-00) for further information regarding the use of robust statistics.

During the CS&T baseline definition procedure, the software establishes cytometer specific reference settings by running the standardized CS&T beads. The CS&T reagent contains equal concentrations of three dyed polystyrene beads differing in relative fluorescence intensity: dim, mid, and bright. The purpose of these baseline settings is to ensure that during characterization the dimmest CS&T bead measurements are not dominated statistically by electronic noise or optical background. Processing CS&T bead data collected at these settings allows the CS&T algorithms to accurately characterize the cytometer's performance. Although CS&T beads can be used to capture and recreate a variety of application settings, beads are generally poor surrogates when it comes to determining what various cell based application settings ought to be. Thus, application settings should be established using cells representative of those that will ultimately be used. To establish good application settings for the flow analysis of a broad range of any particles in suspension, be they beads, cells, or anything else, the general approach is to adjust each detector in such a way as to ensure:

- The dimmest populations have a minimal (acceptable) contribution from electronic noise.
- The brightest (stained) sample is within the linear range of the detector.

The CS&T software uses these general principles to establish the baseline target voltages for the CS&T beads. These principles are especially helpful in setting up to resolve dim cell populations.

Working with highly autofluorescent cells

When creating application settings using highly autofluorescent cells or those exhibiting high nonspecific binding (binding of fluorescent antibody to “negative” populations), the dominant signal contributions at the low end are going to be from the cells themselves rather than any (relatively small) contributions from electronic noise or background. In both these situations, the user should refrain from indiscriminately lowering PMT voltages to place unlabeled cells at the lowest end of the scale, since these levels might be biologically relevant.

When encountering high levels of cellular autofluorescence or nonspecific binding, focus rather on the overall dynamic range of each detector, considering the resolution of all application-relevant populations. For example, CD4 T cells stained with a particular lot of antibody would likely fluoresce at a fairly constant level. However, when working with very autofluorescent cells, it is useful to find a representative positive sample, such as lymphocytes with a normal to dim autofluorescence background, to use in the creation of application settings based on a similar expression level for that marker.

Biological Dynamic Range of Fluorescence Intensity

The primary goal of adjusting fluorescence detector voltage (gain) is to ensure that two fundamental requirements are properly met for every cell based immunofluorescence application:

1. There are no undesirable effects from electronic noise contributions of the system to the measurements of any dim cells that might be present.
2. The voltage gain of each detector must place the populations within the linear dynamic range, given the brightness of the populations to be measured.

When voltage gains are set too low, electronic noise begins to dominate dim cell measurements, and slightly positive populations might not be well resolved from unstained cells. Conversely, when voltage gains are set too high, very bright fluorescent markers might exceed the linear range of a detector, causing errors in spillover calculations and inaccurate compensation.

Part 1. Characterization of a Cytometer

This part summarizes the activities required for the initial measurements for cytometer characterization, including creating a standard configuration, generating baseline a report, and compiling the data needed to create application settings.

Creating a Standard Configuration and Generating a Baseline Report

The first step in standardizing applications within or among labs is to create a standard configuration that can later be exported, shared, and imported across cytometers to be standardized. This hardware configuration should match on all cytometers to be standardized, including the lasers, filter/mirror sets, and fluorochromes used on each detector. The fluorochromes should have the same names in both BD FACSDiva and BD CS&T software.

Use an optimal window extension for a given cytometer platform to allow the system to focus on the most advantageous portion of the detector signal (ie, data). The optimal window extension should be determined by:

- Running a CS&T daily performance check for the existing configuration to ensure that all performance parameters, especially the time delays, are optimal
- Ensuring that current CS&T settings are used in the experiment
- Setting the threshold appropriately for the samples to be run
- Running a brightly stained cell or particle representative of the largest cell or particle to be run
- Adjusting (usually lowering) the window extension in intervals of one until the bright peak median fluorescence intensity (MFI) is at its maximum

For example, if the value observed when the MFI began to decrease was 2.0, then the window extension used would be 3.0. Keep in mind that for instruments for which sheath pressure varies with the level in the sheath tank (BD™ LSR II and BD LSRFortessa™ cytometers) the sheath level should be tightly controlled to maintain the time delays used between lasers when determining the optimal window extension. If this is not possible, leave the window extension at the default value (see BD Technical Bulletin 23-11552-00, *An Introduction to Window Extension on Digital Flow Cytometers*).

Once the preceding adjustments are completed, users can create a modified configuration and set it as the active configuration, using the CS&T module of BD FACSDiva software. A new baseline and daily performance check should then be performed prior to creating application settings (Figure 1).

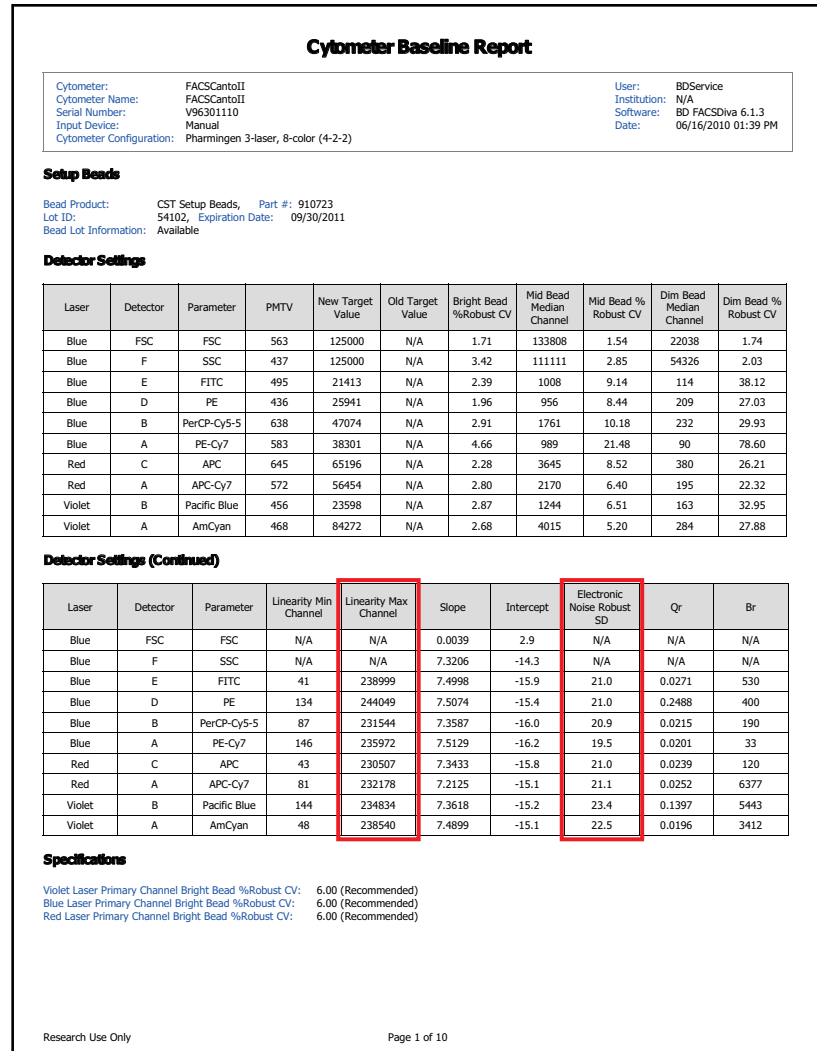


Figure 1. An example of a BD Cytometer Baseline report.

Record the electronic noise robust standard deviation (rSD_{EN}) and the linearity max channel value from the Baseline report to use for creating application settings.

Compiling Data Needed to Standardize Application Settings

In a laboratory that has multiple sites and/or cytometers, a Cytometer Baseline report needs to be generated for each cytometer. Compare the linearity and the rSD_{EN} values (see Figure 1) on each report to ensure that application settings across all the cytometers are robust. The reported highest rSD_{EN} and the Lowest Linearity Max Channel value for each detector from all the Cytometer Baseline reports are used to create a “virtual cytometer” against which all the other instruments will be standardized. These virtual cytometer values are used to create $rSD_{Dim Cells}$ target values and linearity maximum values for the creation of application settings and bright CS&T bead target values.

To ensure that the settings fall outside the electronic noise of each cytometer, an added calculation is required. First, the results of the Cytometer Baseline report (Figure 1) need to be exported to a Microsoft® Excel® spreadsheet. Figure 2 shows an example of the exported Baseline report results. An additional column is created for calculating 2.5 times the robust standard deviation of electronic noise (rSD_{EN}). These values become the new target values that will be used to

create application-specific settings. This calculation will be used when adjusting PMT voltages to create the application settings.

Laser	Detector	Parameter	Linearity Min Channel	Linearity Max Channel	Slope	Intercept	Electronic Noise Robust SD	Qr	Br	2.5 x Electronic Noise Robust SD
Blue	FSC	FSC	N/A	N/A	0.0039	2.9	N/A	N/A	N/A	
Blue	F	SSC	N/A	N/A	7.3206	-14.3	N/A	N/A	N/A	
Blue	E	FITC	41	238999	7.4998	-15.9	21	0.0271	530	52.5
Blue	D	PE	134	244049	7.5074	-15.4	21	0.2488	400	52.5
Blue	B	PerCP-Cy5-5	87	231544	7.3587	-16	20.9	0.0215	190	52.3
Blue	A	PE-Cy7	146	235972	7.5129	-16.2	19.5	0.0201	33	48.8
Red	C	APC	43	230507	7.3433	-15.8	21	0.0239	120	52.5
Red	A	APC-Cy7	81	232178	7.2125	-15.1	21.1	0.0252	6377	52.8
Violet	B	Pacific Blue	144	234834	7.3618	-15.2	23.4	0.1397	5443	58.5
Violet	A	AmCyan	48	238540	7.4899	-15.1	22.5	0.0196	3412	56.3

Figure 2. Example of an exported Cytometer Baseline report in Excel.

The additional column on the right shows the results of the $2.5 \times rSD_{EN}$ target value calculation using the following calculation: $2.5 \times \text{Electronic Noise} = 2.5 \times rSD_{EN}$ for each detector listed (except for FSC and SSC).

Part 2. Rules Used to Create Application Settings

BD Biosciences has developed a process in which application settings can be systematically created using three simple rules derived from key CS&T baseline information for each detector on the cytometer. The following rules are used to create the application settings:

Rule 1.

For each detector, determine a minimum PMT voltage setting for the appropriate unstained cells of interest or representative particles. The minimum acceptable voltage for each detector is set so that the resulting rSD of the unstained cells is approximately 2.5 times the rSD_{EN} . This ensures that the electronic noise does not interfere with the measurements at the low end of the scale (see Formula 1).

$$\text{Minimal } rSD_{\text{Dimmest Cells}} = \frac{rSD_{\text{Electronic Noise}}}{\sqrt{0.15}} \approx rSD_{\text{Electronic Noise}} \times 2.5$$

Formula 1. This general rule is derived by adjusting the PMT voltage so that the contribution from electronic noise is no more than about 10% to 20% of the total measured variance of the dimmest particles to be measured. This guarantees that the voltage settings will be sufficient to resolve any dim populations. The formula works because the standard deviation (σ , or SD) is the square root of the variance (σ^2 or SD^2). Once the PMT voltages are set as described, the dimmest cells should have their measured rSD be approximately $2.5 \times rSD_{EN}$ at the lowest acceptable voltage.

An important consideration when setting the minimum PMT voltages is the window extension setting, which can have a significant impact on how much electronic noise is included. If it is set too large, the window extension can unnecessarily contribute to significant noise in the data, and a loss of resolution sensitivity.

Rule 2.

Make sure there is a good and appropriate use of the dynamic range for each detector. The highest (brightest) positive populations should remain within the linear range of the detector. There should also be some extra room at the top of the scale for higher than expected expression levels. For example, if a two-fold increase in expression is possible, then verify that the signals of the brightest population to be measured do not exceed approximately one half the maximum acceptable values, which is the maximum linearity range in the Cytometer Baseline report (see Figure 1).

Rule 3.

Rule 2 takes precedence over Rule 1. If the positives are so far up the scale using the minimum acceptable voltage that they are either in the nonlinear range or going off scale, then the voltage should be lowered according to Rule 2 regardless of where the negatives ultimately reside. All populations of interest must be in the linear range of each detector to ensure that spillover between all detectors is correctly calculated. Spillover errors lead to poor resolution and incorrect data.

Part 3. Creating the Application Settings

This part describes the processes of running cells to create application settings, making and verifying any adjustments, and saving the settings for future use.

Step 1

To create application settings, first unstained cells are run on the cytometer and voltages adjusted until their rSD value is close to the target value for each parameter (see Figure 3). Because the transient rSD calculations per second during acquisition might not contain enough total events for an accurate estimate, it is best to record data files with at least 500 to 2,000 gated events for a reliable rSD calculation. After that, estimate the required voltage adjustment in a worksheet, create a new tube, and make the estimated adjustment. Record another file to reassess or confirm that the target value has been reached. Repeat this process until the optimal target values are obtained.

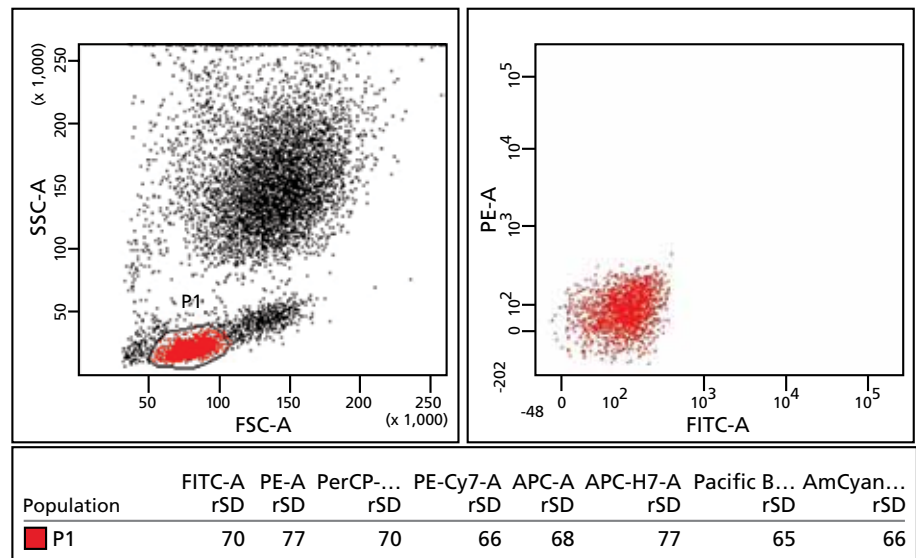


Figure 3. The unstained cells are acquired and the voltages are adjusted until their target rSD ($2.5 \times rSD_{EN}$ from the Cytometer Baseline report) is reached.

Compare the rSD_{EN} target values calculated in the Excel spreadsheet for each fluorescence parameter for the population of interest (in this example, lymphocytes; P1). Adjust the PMT voltages until the target rSD is reached. Based on the calculations in the Excel spreadsheet (Figure 2), all the PMT voltages would need to be decreased to achieve the calculated $2.5 \times rSD_{EN}$ in this example. The resulting PMT voltages become the minimum acceptable voltages and should not be decreased unless there is a compelling need to do so after performing the next step.

Step 2

After determining and setting the minimum acceptable voltages, verify that the dynamic range is appropriate and that highly positive cell populations still are within the linear range for each parameter. According to Rule 2, ensure that the brightest population falls below the maximum linearity value. In addition, any bright populations with very large CVs (very broad distributions) must have their PMT voltages lowered so that the brightest events or MFIs remain within the linear range of the detector (see Rule 3).

Step 3

Once the PMT voltages have been adjusted to satisfy the rule set, these settings can become application settings with recognizable names for future reference. An example could be *Lymphocyte Application Settings mm_dd_yy*. The application settings can be applied to the open experiment in BD FACSDiva software. The application settings are automatically updated based on the results of the daily performance check.

Using Application Settings over Time

Application settings are designed to work only with the baseline definition for which they were created. If a new baseline is created that also requires new CS&T median target values, users will need to create or re-create application settings from CS&T bright bead target values. When changing to a new lot of CS&T beads, use the Reset Target Values function in the CS&T module to keep the existing baseline and to maintain consistent median target values.

Note: Use the original lot of CS&T beads used for baseline definition in the Reset Target Values procedure.

Part 4. Cytometer Standardization

The goal for cytometer standardization is to be able to generate directly comparable results across cytometers with the same or different optical configurations. This is necessary when two or more cytometers will run the same assay, eg, in the same facility, or across multiple facilities, or for collaboration between facilities. To generate comparable results across cytometers with the same optical configuration, implement a method using standardized bright BD CS&T beads. For cytometers with different optical configurations, a nearly identical method using stained BD™ CompBead particles (BD CompBeads) can be implemented. For either method, the application settings described previously can be used in conjunction with CS&T beads or BD CompBeads to create target values for standardizing the setup of all the cytometers.

Generating Target Values Using the Application Settings

To create application settings from target values with the bright CS&T beads, use a BD FACSDiva global worksheet to create plots for forward scatter (FSC) vs side scatter (SSC), and histograms for each fluorescence parameter to be standardized. Adjust the FSC and SSC so that only CS&T mid and bright bead singlets are captured.

Note: It is helpful to use the Zoom In function in BD FACSDiva software to discriminate between the different populations.

The fluorescence parameters' histograms should display only the identified bright bead population in the FSC vs SSC dot plot. Create Interval gates on the global worksheet to include the bright bead population for each fluorescence detector. The MFI values should be displayed for each population in a statistics view. These

MFIs serve as the bright bead target values for each respective parameter. These target values, along with the CS&T bead lot number, are then recorded in a text box on the global worksheet (see Figure 4). This worksheet can be exported as a standard analysis template to other cytometers.

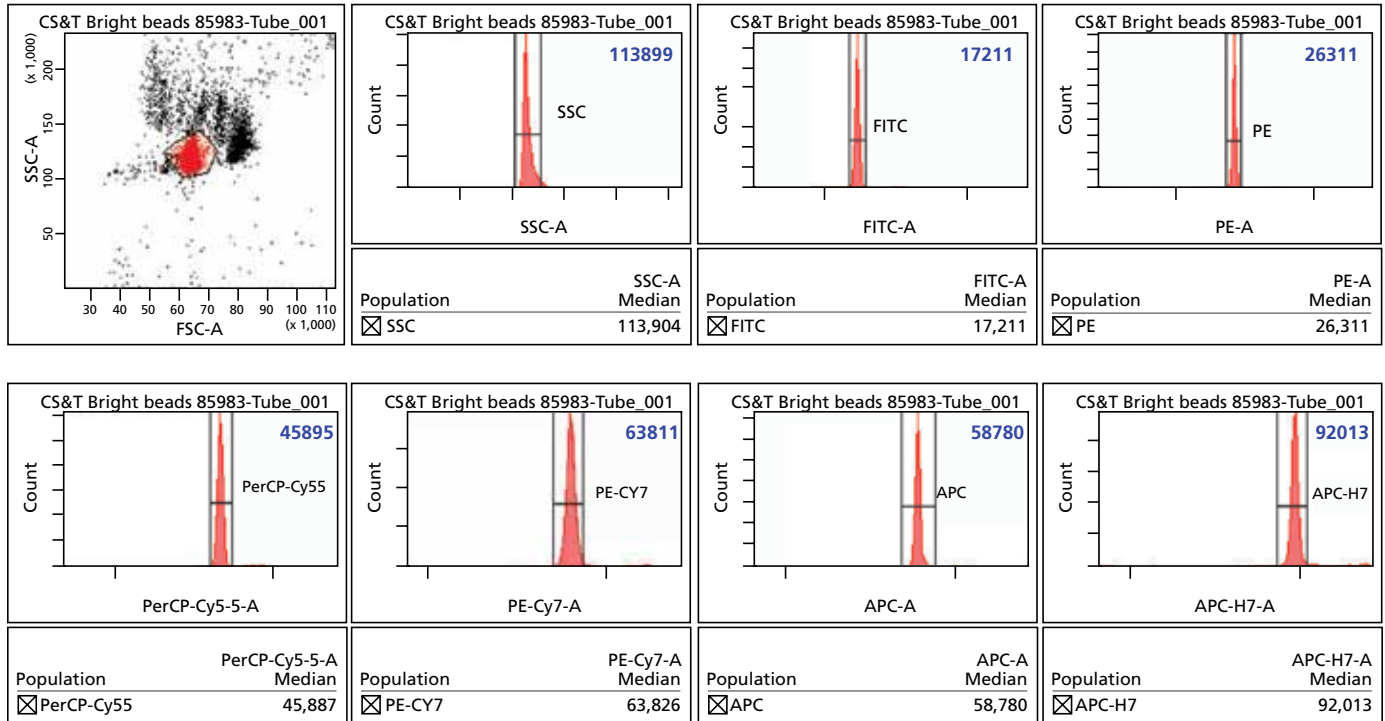


Figure 4. An example of a global worksheet when CS&T beads are acquired to create target values.

The CS&T bright beads are gated in the FSC vs SSC plot and are shown in the histogram plots (the Zoom In function was used to show the bright bead population). The target value for each parameter is displayed in the statistics view by showing the median within the histogram gates. The CS&T bead lot must be recorded on the template for future reference.

Note: These target values are for demonstration purposes only and might not reflect the performance of any specific cytometer.

Standardizing Cytometers with the Same Optical Configuration

BD CS&T beads are used to standardize cytometers when their optical configurations are the same. Users can expedite creating the identical configurations on multiple cytometers by selecting the configuration used to create the application settings and exporting it in a CSV (comma-separated value) format to the D:\BDEExport\Instrument folder. The configuration can be imported on other cytometers' workstations from the CS&T module in BD FACSDiva software.

The standardization analysis worksheet can be exported as an analysis template to the D:\BDEExport\Templates\Analysis folder and then transferred to the workstations of the other cytometers as a BD FACSDiva analysis template.

After importing the template to another cytometer to be standardized, create a new experiment and use the imported analysis template to create a global worksheet. The Interval gates on the worksheet are used as target values for each fluorescence parameter when acquiring the same lot of CS&T beads (Figure 4). Adjust the PMT voltages until the populations are at their respective target values. After the voltages have been determined, these settings are used to create application settings. Figure 5 shows an example of data consistency between cytometers after this standardization protocol has been applied.

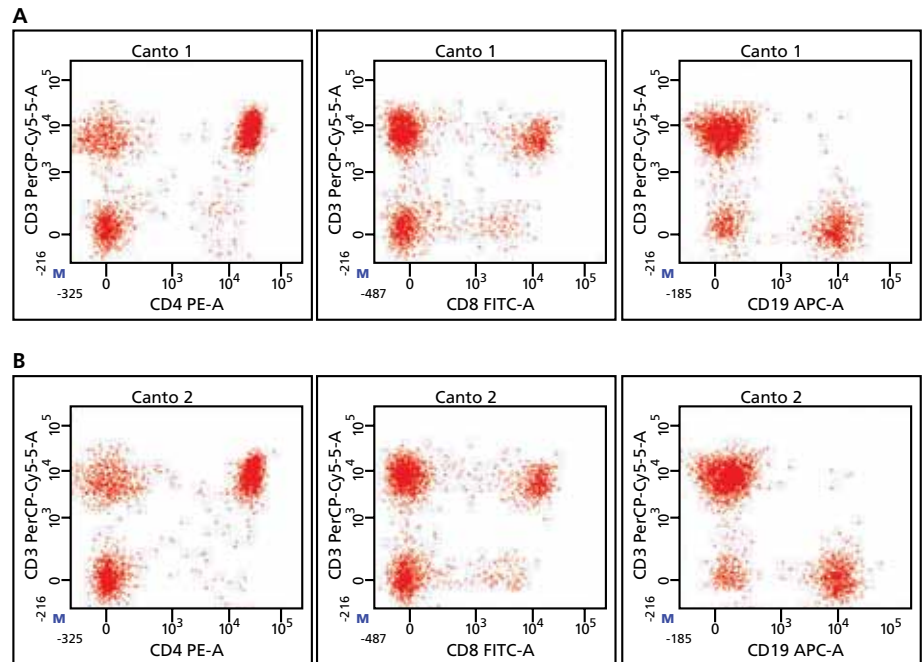


Figure 5. Data demonstrating consistency between BD FACSCanto™ II cytometers after the instrument standardization.

BD FACSCanto II (A), BD FACSCanto II (B). Note that the manual biexponential display (M) was used to compare the results from the different cytometers.

Standardizing Cytometers with Different Optical Configurations

When standardizing cytometers with different optical configurations, a virtual cytometer is created by taking the highest SD_{EN} and the lowest linearity max channel values for each detector of any of the cytometers to be standardized. Also, BD CompBeads stained with the appropriate fluorochrome conjugates for each detector are used instead of BD CS&T beads to establish standardized target values and application settings. The same lots of BD CompBeads and fluorochrome conjugates must be used throughout the standardization process for all cytometers. Example data demonstrating consistency between multiple platforms after cytometer standardization is shown in Figure 6.

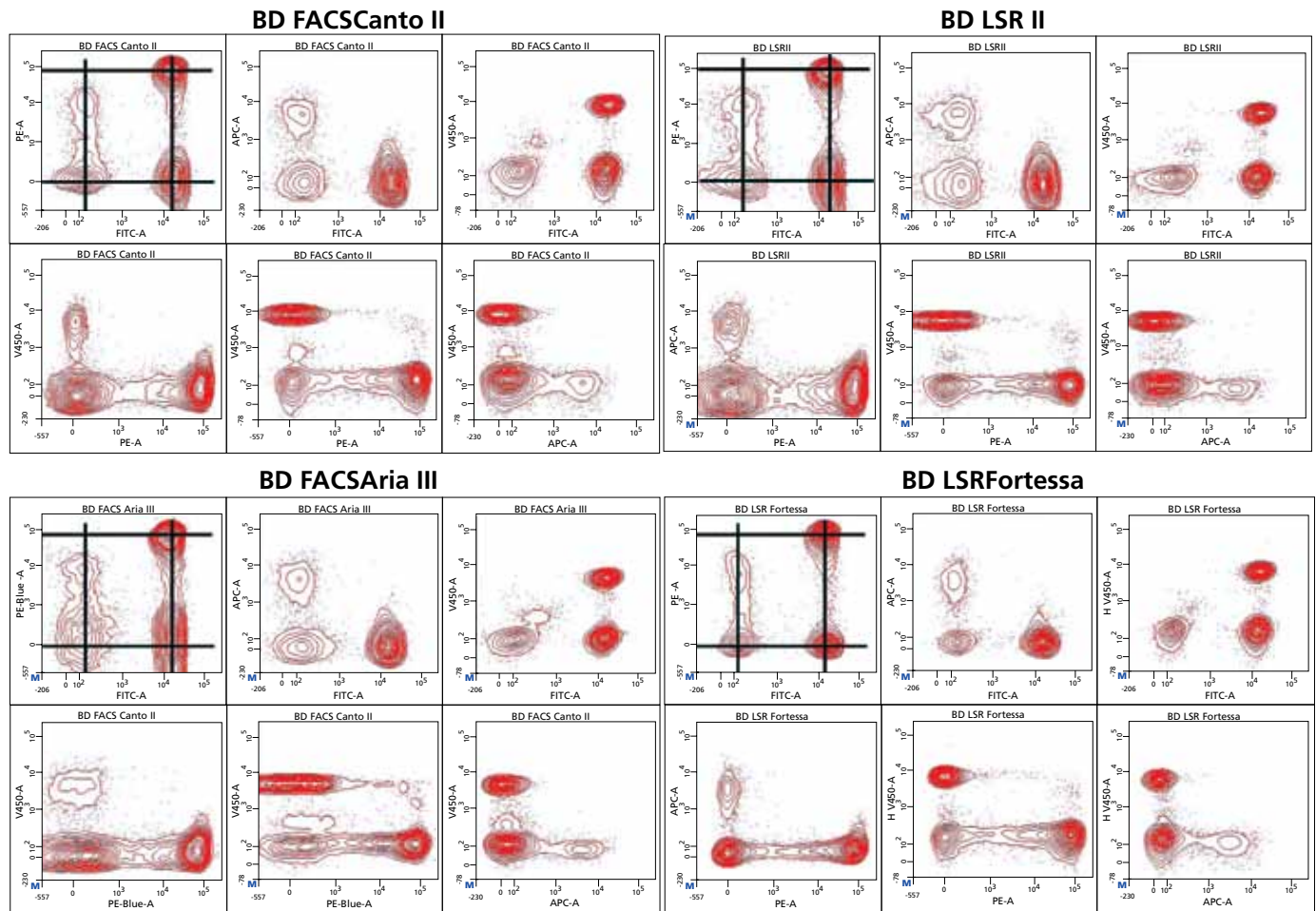


Figure 6. Data demonstrating consistency between different cytometers with different optical configurations after the standardization procedure.

Part 5. Verification of Standardization Accuracy

Before starting the verification procedure, ensure that the cytometer is clean and all air is purged from the fluidic filters, fluidic lines, and flow cell. Then, run a performance check and ensure that it passes before beginning verification.

Verifying Standardization

The original lots of BD CS&T beads used for the initial standardization can be rerun on each cytometer using the standardization templates on each cytometer. Record the MFIs for 20 replicate runs. The MFIs should not vary by more than two standard deviations (2SD or within the 98th percentile rank) over time from the original target values. To establish 2SD, monitor 20 runs over time. If a cytometer's MFIs vary by more than 2SD, adjust the PMT voltages to bring the MFIs back to the standardized target values displayed in the text boxes.

Appendix: Step-by-Step Standardization of BD Cytometers Using BD FACSDiva Software v6.0 or Later

Creating Application Settings

Preparing to create application settings

1. Start BD FACSDiva software and connect to the CS&T module.
2. Make sure that the baseline is current and the daily performance check has been performed and has passed.
3. Export the Cytometer Baseline report results by right-clicking on the report and selecting **Export**.
4. Open the report in a Microsoft Excel spreadsheet.
5. Add a column at the right called $2.5 \times SD_{EN}$ and perform a calculation of $2.5 \times SD_{EN}$ for each fluorescence parameter row using the Excel calculation shown in Figure 2.
6. Print the Excel worksheet.
7. Exit the CS&T module and select **Use CST Settings** in the dialog that opens.
8. Create a new experiment in the Browser and name it *Application Settings mm_dd_yy*.
9. Verify that the CS&T settings are current (right-click the experiment-level Cytometer Settings and select **Apply Current CST Settings**).
10. In the Inspector, select the parameters that will be used in the application.

Creating the Application Settings

1. Create a dot plot for FCS vs SSC and histogram plots for each fluorescence parameter on a global worksheet.
2. Draw a P1 gate for the CS&T bead singlets in the FSC vs SSC dot plot, select all the fluorescence histograms, then right-click and select **Show Populations > P1**.
3. Create a statistics view to show the rSD of the negative population for all the fluorescence parameters by right-clicking the population hierarchy and selecting **Create Statistics View**.
4. For each fluorescence parameter, verify that negative populations have an $rSD \approx 2.5 \times SD_{EN}$ (compare the values in the statistics view to the printed Excel worksheet) by acquiring unstained cells. Since BD FACSDiva software cannot calculate the rSD of a population during acquisition, do not attempt to obtain an rSD in the acceptable range by adjusting the PMT voltage during acquisition.
5. Record data, check the rSD in that statistics view, go to the next tube, adjust the voltages as needed, record, and repeat.
6. Using the same voltage settings, verify that the median of the highest positive population is lower than the linearity max channel.
7. If the positives are too high, lower the voltage to get the highest positive population within the linear range, regardless of the negative population rSD.

Saving the application settings

1. After the rule set conditions are met, save the application settings by right-clicking **Cytometer Settings** in the Browser toolbar and selecting **Application Settings > Save**.
2. To use the application settings in the future, right-click **Cytometer Settings** in the Browser toolbar and select **Apply Application Settings**.

Standardization of Cytometers with the Same Optical Configuration

Make sure that all the cytometers to be standardized have the same optical configuration (lasers, laser powers, mirrors, and filters). If the instruments have the different optical configurations, first read the instructions under Standardization across different cytometers with different optical configuration and then follow this procedure.

Note: For cytometers with the same optical configuration, the same lot of BD CS&T beads must be used throughout the standardization process for each instrument.

Creating the initial application settings

1. Connect to the CS&T module and make sure that the baseline and the daily performance checks have been performed for each cytometer to be standardized.
2. Determine the target values for standardization by comparing the linearity and the electronic noise robust SD from the Cytometer Baseline reports for each instrument. The lowest common denominator (the highest SD_{EN} and the lowest linearity max channel reported) for each parameter and cytometer need to be determined. These values will become the virtual cytometer target values.
3. Calculate the $2.5 \times SD_{EN}$ for each fluorescence parameter by using the virtual cytometer values.
4. On one of the cytometers to be standardized, create a new experiment in BD FACSDiva software and name it *Cytometer Standardization*.
5. To apply the current CS&T settings to the new experiment, right-click **Cytometer Settings** in the Browser toolbar and select **Apply Current CST Settings**.
6. In the Inspector, select the parameters that will be used in the application.
7. Create a dot plot for FCS vs SSC and histogram plots for fluorescence parameters on a global worksheet.
8. Draw a P1 gate for the population of interest in the FSC vs SSC dot plot, select all the fluorescence plots, and right-click and select **Show Populations > P1**.
9. Create a statistics view to show the rSD of the negative population for each fluorescence parameter.
 - a. Right-click the population hierarchy and select **Create Statistics View**.
 - b. Right-click on the statistics view and select **Edit Statistics View**.
 - c. Click the **Header** tab and clear all the boxes.
 - d. In the **Populations** tab, select the **P1** checkbox and clear all the other boxes in the tab.
 - e. Clear all the boxes in the **Statistics** tab, select only the rSD fluorescence parameter boxes, and click **OK**.

10. Create application settings following the protocol described in the preceding Creating Application Settings section by using the virtual cytometer target values for rSD_{EN} and linearity maximum, and by acquiring unstained cells. If you already have created application settings, apply them by right-clicking the experiment-level Cytometer Settings in the Browser and selecting **Application Settings > Apply**. Apply the application settings from the catalog for which you want to record standardization targets.

Standardizing the first cytometer

To create the target values for the standardization of the other cytometers, create a global worksheet where histogram plots are shown for each fluorescence parameter to be standardized.

1. In the Acquisition Dashboard, set the flow rate to low and the number of events to acquire to 20,000.
2. Using this acquisition template and the application settings, acquire data from CS&T beads with a known lot number and adjust the FSC and SSC if needed so that the mid and bright beads are captured. If needed, use the Zoom In function to show the different bead populations.
3. If needed, back gate from the bright bead peak in another fluorescence parameter to correctly identify the bright bead population. It is helpful to use the Zoom In function in BD FACSDiva software to discriminate between the different populations.
4. Acquire and record the tube.
5. Gate the bright bead population in the FSC vs SSC plot and show the bright beads in the histogram plots for each parameter to be standardized.
6. Create Interval gates to include the bright bead peaks in the histograms.
7. Display the median values of the bright bead population in the statistics view. These values will be used as target values when standardizing other cytometers.
8. Record the median value for each detector in the text box on the matching parameter histogram.
9. Export the global worksheet as an analysis template and name it *Bright Bead Targets_assay name*: Right-click on the global worksheet and select **Export > Analysis template**. The analysis template is saved in D:\BDExport\Templates\Analysis.

Standardizing the other cytometers

1. Import the analysis template to the computer connected to the next cytometer to be standardized, for example, by using a USB memory stick.
2. Save the template file in D:\BDExport\Templates\Analysis.
3. On the second cytometer, click the **New Experiment** button on the Browser toolbar.
4. Verify that the CS&T settings are current (right-click on the Cytometer Settings and select **Use Current CST settings**).
5. Under **New Experiment > Apply Analysis Template**, select the analysis template you have created and saved from the Analysis Template folder on the D drive.
6. In the Acquisition Dashboard, set the flow rate to low and the number of events to acquire to 20,000.
7. Acquire CS&T beads with the same lot number as the beads that were used in the previous cytometer.

8. Using the Interval gates in the analysis worksheet, adjust the PMT voltages in the **Instrument Control** panel to hit the target median value listed in the text box for each parameter. Do not move the gates; use them as targets.
9. Record the tube and check the statistics to make sure the target median values have been reached.
10. Right-click on the Cytometer Settings and select **Application Settings > Save**.
11. Name the application settings to match the name on the base cytometer for the application being standardized.
12. Repeat steps 4 to 11 for each cytometer to be standardized.
13. Each time the application settings are applied in each cytometer, the voltages are updated according to the daily CS&T performance check.

Standardization Across Different Cytometers with Different Optical Configurations

If the cytometers to be standardized have different optical configurations (lasers, laser powers, mirrors, and filters), you can use the same procedure as that for standardizing instruments with the same configuration, with the following exceptions:

- Instead of using BD CS&T beads, run the same lot of BD CompBeads throughout the standardization process for each cytometer.
- When you have completed the standardization, the record bright bead target values for each different optical configuration so that application settings can be recreated and instrument performance monitored over time. The application settings may also be used to standardize setup for additional cytometers with the same optical configuration.

Transferring Bright Bead Targets Values to New BD CS&T Bead Lots

Over time, new BD CS&T bead lots will be required to generate the bright bead target values. Use this procedure to generate bright bead target values for each succeeding lot of CS&T beads so that you can indefinitely regenerate application settings as needed.

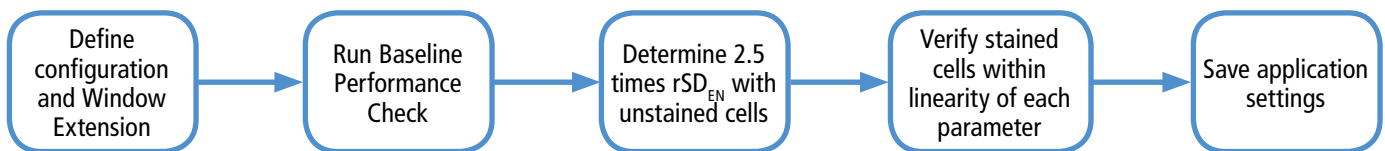
1. Start the CS&T module and make sure the daily performance check has been performed with the current CS&T bead lot.
2. Create a new experiment in the Browser and name it *Bright Bead Target Transfer*.
3. Verify that the CS&T settings are current (right-click on the Cytometer Settings and select **Use Current CST Settings**).
4. Right-click on the Cytometer Settings and apply the application settings for the bright bead targets to be transferred.
5. When the dialog opens, click **Overwrite**.
6. Right-click the global worksheet icon, navigate to D:\BDEExport\Templates\Analysis, and apply the Bright Bead Targets worksheet that was previously saved.
7. In the Acquisition Dashboard, set the flow rate to low and the number of events to acquire to 20,000.
8. Acquire data from the new lot of CS&T beads and adjust the FSC and SSC if needed so that the mid and bright beads are captured.
9. Adjust the bright bead gate so that only the median of the bright bead population is observed in the statistics view.

10. Record the tube.
11. Enter the new values for the MFI of each detector in the appropriate text boxes.
12. Enter the date and new CS&T bead lot number in the appropriate text boxes as shown in Figure 4.
13. Right-click the global worksheet icon and export the updated Bright Bead Targets worksheet. Rename the sheet so as to not overwrite the original worksheet from the previous bead lot.

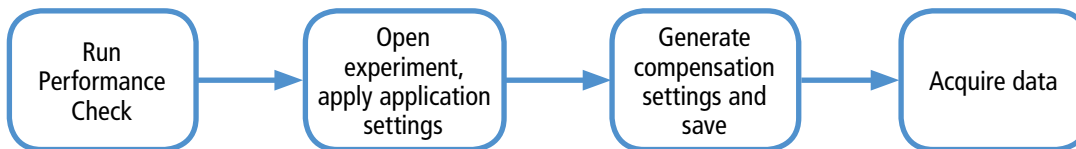
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Initial Setup of a Cytometer



Daily Workflow



¹ Note that although the term channel is used within the CS&T report to denote the maximum acceptable signal level (channel has become synonymous with signal level for many in the flow community), all raw BD digital pulse area FCS 3.0 data is actually composed of 32-bit single precision IEEE floating point numbers. In contrast to unsigned integer data in other systems, there are actually no integer channels in BD digital data, even though the maximum declared range is equivalent to 218 (262,144). The resolution is far greater than 18 bits, since in firmware, pulse area measurements are summed in a 24-bit register using 4 fractional bits, then converted to a single precision float before being sent to the software. As a result, technically there are no channels.

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