Compensation tools on the BD FACSLyric™ Flow Cytometer

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A. Introduction to compensation in multicolor flow cytometry

The use of antibody-labeled fluorochromes in flow cytometry assays requires a correction for the spillover of one fluorochrome signal into another detector. This correction is called compensation. It is essential in multicolor flow cytometry to correct fluorescence spillover. As laboratories move into higher parameter flow cytometric assays, proper compensation becomes more challenging for generating accurate data. To properly auto-calculate compensation, an appropriate set of single-color controls is critical for success. These controls are used to measure fluorescence spillover in each channel. The software's compensation algorithm then removes this spillover from each fluorescent parameter and calculates a compensation matrix. When this matrix is applied to fully stained samples, fluorescence spillover is corrected in every channel.

Although this document will not discuss panel design, it is important to consider the density of target antigens, the brightness of the fluorochrome and the spread of the fluorochrome into other channels when matching antibodies and fluorochromes. Refer to Figure 3 for common fluorochromes that can be used on the BD FACSLyric™ Flow Cytometer and their respective excitation and emission.

Verifying compensation:

Each laboratory must establish their own procedures for verifying that compensation is appropriate (Figure 1). Mathematical verification of compensation can be performed by observing that the median fluorescence intensity (MFI) of the positive and negative populations are within a certain percentage, as established by the laboratory. Visual verification involves looking for a symmetrical shape surrounding the MFI of the negative value when compared to the MFI of the positive population. The axes need to be set to biexponential display with the same scaling on each plot (Figure 2). Properly verifying compensation regularly provides an additional level of quality control to ensure data integrity.

Figure 1: An example worksheet with a biexponential scale for checking compensation using the median X-axis values of the positive and negative populations.

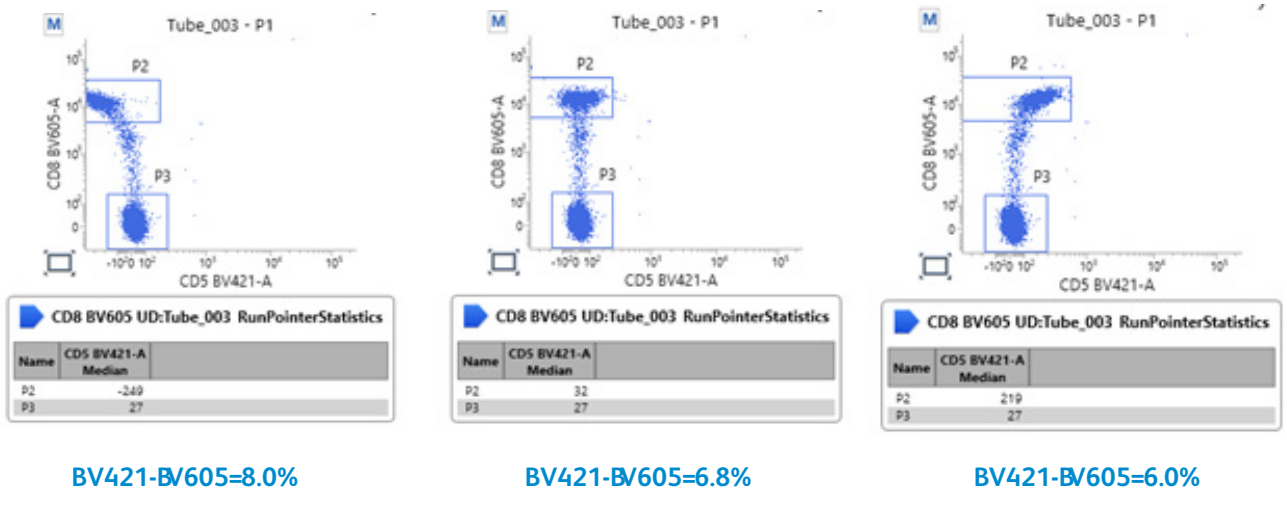
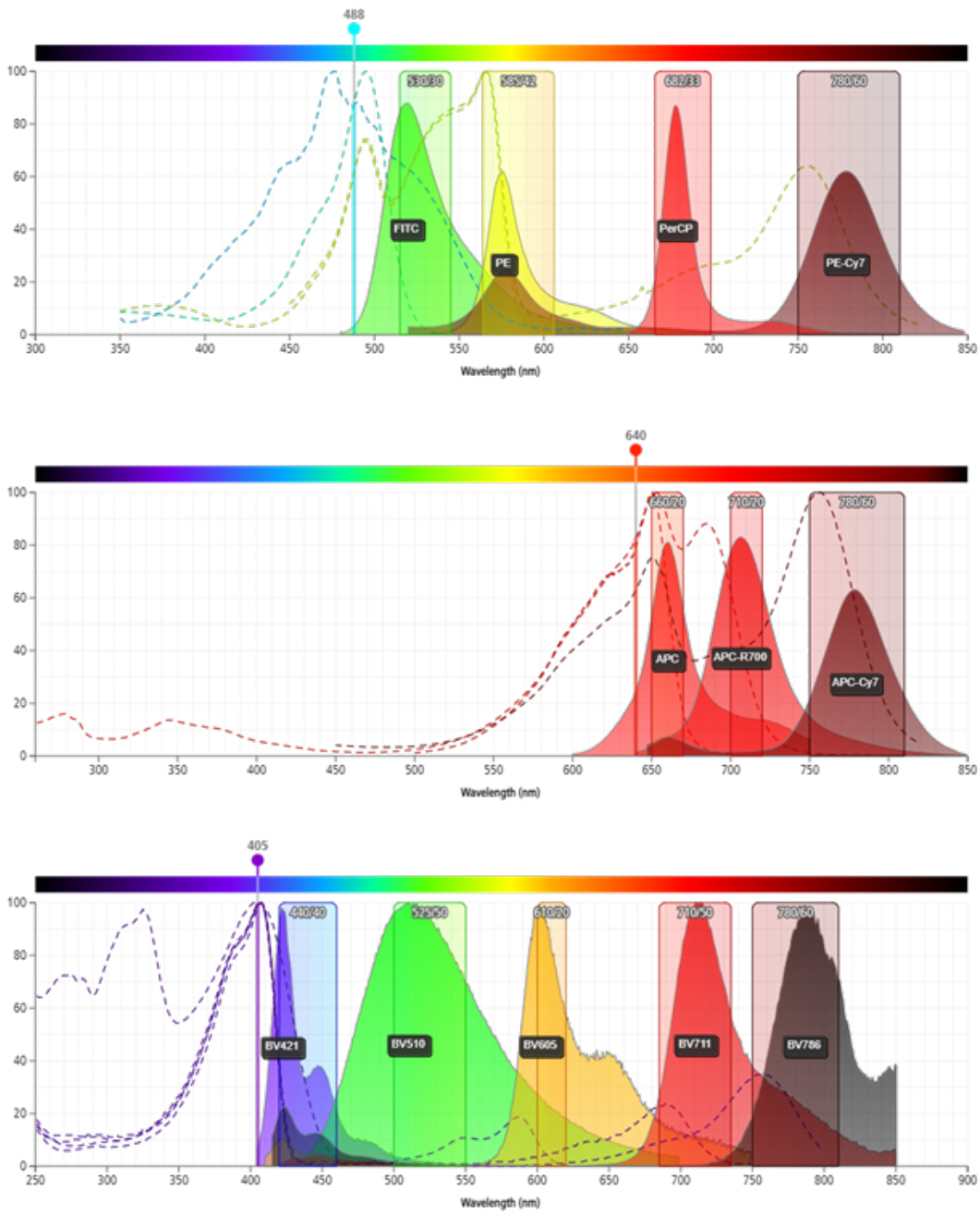


Figure 2: An example worksheet with a biexponential scale showing overcompensation, appropriate compensation and under compensation using the median X-axis values of the positive and negative populations.



Source: https://www.bdbiosciences.com/en-us/resources/bd-spectrum-viewer

Figure 3: Demonstration of fluorescent excitation (dotted lines) and emission (solid lines with colored area under the curve) spectra for typical fluorochromes used on the BD FACSLyric™ Flow Cytometer. Rectangles show the range of light passing through band pass filters to a detector being recorded as signal.

B. Calculating compensation and optimizing settings on the BD FACSLyric™ Flow Cytometer

Compensation is calculated in the BD FACSuite™ Application by measuring single-color controls for each channel and every fluorochrome used in that channel. The mathematical algorithm uses inverse matrix algebra and is the same algorithm used in BD FACSDiva™ Software.

Compensation values are the ratio of fluorescence measurements in the spillover detector divided by those in the primary detector, and therefore they have no units. When cytometers with analog electronics systems (e.g., the BD FACSCalibur™ Flow Cytometer) were in use, compensation was applied to the data using actual signal subtraction in the electronics of the instrument. Then, having a high compensation value, or a value near 100%, indicated some kind of data error. Since the invention of digital flow cytometers (e.g., the BD FACSCanto™ Flow Cytometer, BD FACSLyric™ Flow Cytometer), compensation is calculated using inverted matrix algebra. That matrix is then applied to the data in the software itself and not the electronics of the instrument. Therefore, it is now common to see compensation values above 100 without it being an indication of data error. This is also why compensation can now be adjusted post acquisition.

A unique feature of the BD FACSLyric™ Flow Cytometer is the ability to adjust PMT voltages after compensation matrix calculation. This is achieved by normalizing PMT voltages into Tube Target Values. This normalization allows compensation to be recalculated each time the PMTVs are changed. Therefore, it isn't necessary to re-acquire compensation controls every time a voltage is changed.

Lyse Wash and Lyse No Wash reference settings and BD® FC Beads

The BD FACSLyric™ Flow Cytometer uses two built-in reference settings—Lyse Wash (LW) and Lyse No Wash (LNW). LW and LNW reference settings are initially created upon instrument installation by the field service engineer using BD® FC Beads. These reference settings are default settings and are a good starting point, but each laboratory can optimize these settings based on their assay needs.

Each reference setting contains tube settings and spillover values (SOV). Tube settings include MFI targets for determining daily voltage settings. Since tube settings are established using BD® CS&T Beads, it is critical that users upload the correct BD® CS&T Bead Lot file to match the bead lot that is currently in use. When running BD® CS&T Beads during PQC and Assay/Tube Settings Setup (ATSS), the PMTs are adjusted to reproducibly hit MFI target values so that assay performance is consistent from day to day.

Note

Prepared BD® CS&T Beads should be used within 20 minutes after dilution and protected from light to minimize performance issues from possible light degradation.[1] Always refer to the manufacturer's IFU for proper handling of any reagent.

For LW and LNW reference settings, the SOVs are measured using BD® FC Beads and should be updated every 60 days. BD® FC Beads are single-color bead-based compensation controls containing both a positive and negative bead. There is a BD® FC Bead for most of the common fluorochromes used on the 12-color BD FACSLyric™ Flow Cytometer to build a library of spillover values (Table 1).

Table 1: Available BD® FC Beads

FITC
PE
PerCP
PerCP-Cy5.5
PE-Cy7
APC
APC-Cy7
APC-H7
APC-R700
V450
V500-C
BV605
BV421
BV510
BV711
BV786

BV = BD Horizon Brilliant™ Violet V = BD Horizon™ Violet

R = BD Horizon™ Red

C. Optimizing spillover values on the BD FACSLyric™ Flow Cytometer

If the default compensation values with BD® FC Beads are not sufficient for a laboratory developed test, the BD FACSuite™ Application has several options for adding the SOVs required for any assay.

Option 1:

Add fluorochromes to existing LW reference settings.

This option allows the convenience of using BD® FC Beads combined with the flexibility of measuring spillover values from different sources. Adding fluorochromes to existing LW reference settings is used to measure spillover using antibody capture beads or cell-based single-color controls.

Option 2:

Create user-defined reference settings specific to each assay.

This option combines user-defined MFI targets and measured SOVs from user-defined single-color compensation controls. These options along with the default LW and LNW reference settings are listed in Table 2 and will be discussed in greater detail.

Table 2:

Term	Synonym	Definition
Lyse No Wash (LNW) reference settings	Preset MFI targets and SOV	• Default settings determined using normal, lysed no wash whole blood • Generic spillover values measured with BD® FC Beads
Lyse Wash (LW) reference settings	Preset MFI targets and SOV	• Default settings determined using normal, lysed and washed whole blood • Generic spillover values measured with BD® FC Beads.
Add fluorochromes to LW reference settings	Add single-color compensation controls to default settings	• Default MFI targets or user-defined targets • Generic spillover values measured with BD® FC Beads • Additional spillover values measured using BD® FC Beads for additional fluorochromes in a single channel (i.e., adding SOVs for BV421 to the V450 channel) • User-defined spillover values measured with antibody capture beads and cell-based controls for fluorochrome/assay-specific accuracy.
User-defined (UD) reference settings	MFI target values and UD SOVs	• MFI target values determined for a specific assay defined by the user • Measured SOVs for assay-specific compensation controls. These controls can be any combination of BD® FC Beads, antibody capture beads or cell-based single-cell controls

Adding fluorochromes to LW reference settings

Sometimes, the generic compensation values in the default LW reference settings are not adequate for a particular combination of reagents in a single tube. The user can use any single-color compensation control (cells, antibody capture beads, BD® FC Beads) to add SOVs for a specific channel or specific reagent lot. Consult the BD FACSLyric™ Flow Cytometer Reference System for step-by-step instructions. Listed are some commonly used single-color compensation controls and the potential pros and cons of each one.

A. BD® FC Beads:

- Dried-down beads bound to fluorochromes
- Single-color controls for the default LW and LNW reference settings on the BD FACSLyric™ Flow Cytometer
- Carefully follow the IFU for instructions for optimal handling.
- Lot-matched bead files are critical for accurate compensation values

Pros:

- Include matched positive and negative bead populations
- Have known and predictable stability if the IFU is followed closely
- Automated gating

Cons:

- Spillover values that may not be accurate for all reagents and lots of reagents
- Are not available for all fluorochromes

B. Antibody capture beads/BD® CompBeads Particles (Figure 4)

- Beads coated with an antibody that binds human fluorochrome-labeled antibodies
- Follow specific manufacturer's protocol for bead preparation[2]
- **Can be used:**
  - When cells are not readily available
  - When cells do not yield enough positive events to calculate compensation
  - For antibodies that stain in a heterogeneous pattern instead of yielding a tight peak
- Staining should mimic panel staining with regard to final reconstitution buffer and BD Horizon™ Brilliant Stain Buffer (BSB).[3]

Pros:

- Can use identical reagents used in the panel for most reagents (see Cons for exceptions)
- Includes automated gating for the bead population (for BD® CompBeads Particles in the BD FACSuite™ Application)

Cons:

- Beads may not bind all species and/or isotypes of antibodies
- Spillover values may vary when compared to cell staining
- Cannot be used for calculating compensation for non-antibodies or dye stains such as 7-AAD, SYTO™ and FLAER

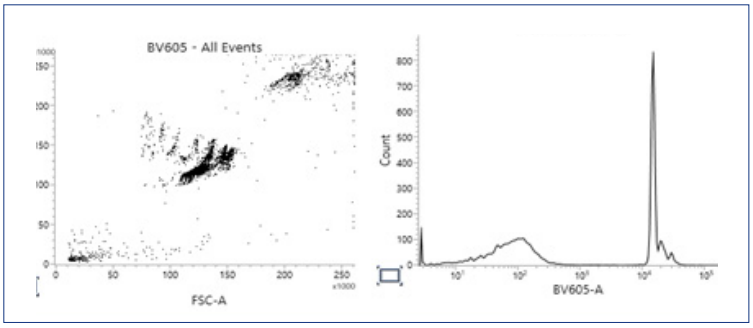


Figure 4: An example of BD® CompBeads Particles as a single-color compensation control run on the BD FACSLyric™ Flow Cytometer.

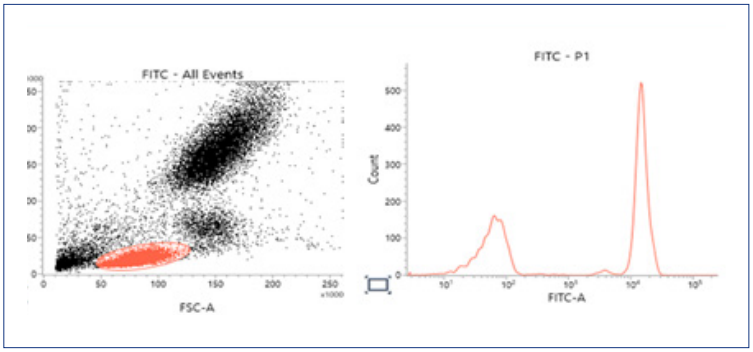


Figure 5: An example of stained cells as a single-color compensation control.

C. Single-stained cells (Figure 5):

- Cell samples can be obtained from fresh specimen or commercially available products
- Staining protocol should be identical to that of the whole panel, including the addition of permeabilization buffers, Brilliant Stain Buffer and final suspension buffer.
- A positive and negative population of the same cell type is needed for accurate compensation calculation as the amount of autofluorescence in different cell types (and between beads and cells) can impact this calculation[4]

  - For example, for measuring antigens on lymphocytes (i.e., CD19 or CD3), one should have a negative population that is a lymphocyte. For a monocyte marker (i.e., CD33), a monocyte without CD33 (CD33-) is the appropriate negative control and this could be achieved by spiking in unstained cells

Pros:

- Single-stained cell compensation controls are the closest to biological samples
- Inexpensive

Cons:

- Time consuming to prepare
- Must have regular access to cells (either normal or abnormal)
- May not be enough positive events to gate (i.e., CD34+ cells in healthy samples)

The choice of compensation controls is particularly important when considering tandem dyes. The spillover values of tandem dyes may vary due to their manufacturing and conjugation processes.[3] Tandem-specific or even tandem lot-specific single-color controls may be needed and can be set up with cells and/or antibody capture beads depending on the unique needs of the laboratory's panels. Tandem dyes can be more susceptible to degradation (due to light, temperature or fixation), potentially causing changes to compensation values over time or under different experimental conditions. This can be controlled by re-measuring SOVs with current lot/open vial of reagents using identical experimental conditions. As more tandem dyes become available, understanding each dye's unique chemistry and stability characteristics can inform users on whether lot-specific compensation is necessary.

Setting up user-defined reference settings:

User-defined (UD) reference settings contain custom tube settings and SOVs measured from user-defined single-color compensation controls. All reference settings are saved in the BD FACSuite™ Application library and can be used to build future assays. UD reference settings can be saved and applied to a tube (or multiple tubes) in an assay. The default LW reference settings can be used in combination with reference settings. In addition, UD reference settings are updated separately from LW and LNW reference settings when the SOVs are remeasured (for example, following major maintenance).

Note

The BD FACSuite™ Application does not require users to update UD reference settings every 60 days so SOV remeasurement frequency should be determined by the laboratory.

D. Quality control of spillover values and compensation matrices

Compensation settings must always be assessed for each assay. It is recommended that compensation is monitored periodically. The method and frequency are up to each laboratory. One way to do this is to create biexponential dot plots to look at every X vs Y combination in each tube on a worksheet (Figure 1). The SOVs can then be verified as appropriate by the laboratory at specific time points determined by the user.

E. Updating and re-determining SOVs

The frequency of updating the SOVs is laboratory dependent. In the case of LW and LNW reference settings, the BD FACSuite™ Application library requires performing an update every 60 days with BD® FC Beads. Updates of user-added compensation controls and UD reference settings must be determined by the laboratory. In addition, when a major maintenance is performed that impacts the optical pathway (e.g., replacing a laser, changing filters), SOVs may need to be re-established.

F. Conclusion

This document combined with the BD FACSLyric™ Flow Cytometer Clinical Reference System provides tools that may be useful when setting up compensation on the BD FACSLyric™ Flow Cytometer. Additional assistance may be obtained from other flow cytometry colleagues and BD Technical Applications Scientists.

References

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2 BD® CompBeads Particles Set product insert. San Jose, CA: Becton Dickinson and Company; 2017. Accessed February 9, 2023. https://www.bdbiosciences.com/content/dam/bdb/products/global/reagents/flow-cytometry-reagents/research-reagents/panels-multicolor-cocktails-ruo/552843_base/pdf/552843.pdf

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