ICCS
International Clinical Cytometry Society

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Title: User's Guide for establishing appropriate Compensation Settings for Lab Developed Tests on the BD FACSLyric™

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Date: January 25, 2023

Preface

This Guide has been written by experienced BD FACSLyric™ users to help laboratories with alternative procedures for spectral compensation using the FACSuite™ RUO software for laboratory developed multi-color panels. These tips and tricks may be helpful when standard procedures yield results that are not optimal. These procedures have been developed in the authors' laboratories, have not been endorsed by BD, and should be independently validated locally prior to implementation in clinical assays. The authors hope that users will be able to use this document as a Guide (Refer to Glossary at end for definition of terms) in conjunction with the BD FACSLyric™ Operator's manual, assistance from BD Technical Application Specialists, and assistance from other flow cytometry colleagues.

Spectral Compensation in Polychromatic Flow cytometry

As laboratories transition to using higher-parameter flow cytometry assays, proper compensation becomes more challenging. The BD FACSLyric™ instrument and software employ software based algorithms to auto-calculate spillover values and create a compensation matrix. Creation of a compensation matrix begins with single-color controls which are used to generate fluorescence spillover values in each channel. The instrument and software's compensation algorithm then subtracts this spillover from each fluorescent parameter and calculates a compensation matrix. The spillover values that define the compensation matrix should be assessed for adequacy using single and fully stained tubes. In some cases, the calculated algorithm using single-color color controls may not be adequate and this module will discuss alternate procedures.

Verifying/checking compensation:

The compensation correction values can be assessed both mathematically and visually, and each laboratory must establish their own procedures for verifying that compensation is correct. Mathematical verification is done by determining median fluorescence intensity or MFI of the positive and negative populations for a given fluorescent parameter and determining that they are similar (the definition of similar is may be an acceptable percent difference established by the laboratory). Visual verification is done by evaluation for a symmetrical shape (commonly referred to as a fishtail, martini glass, trumpet and/or ice cream cone) on a bi-exponential display surrounding the median fluorescent point of the negative value when compared to the median fluorescent point of the positive population for a given fluorescent parameter (Note: this may or may not be at zero). Use of a bi-exponential display facilitates assessment of compensation (**Figure 1**).

Properly verifying compensation regularly provides an additional level of quality control to ensure data integrity. **Figure 2** displays examples of over, appropriate, and under-compensation and both **Figures 1 and 2** show a symmetrical shape surrounding the zero (red dash line in **Figure 1**)

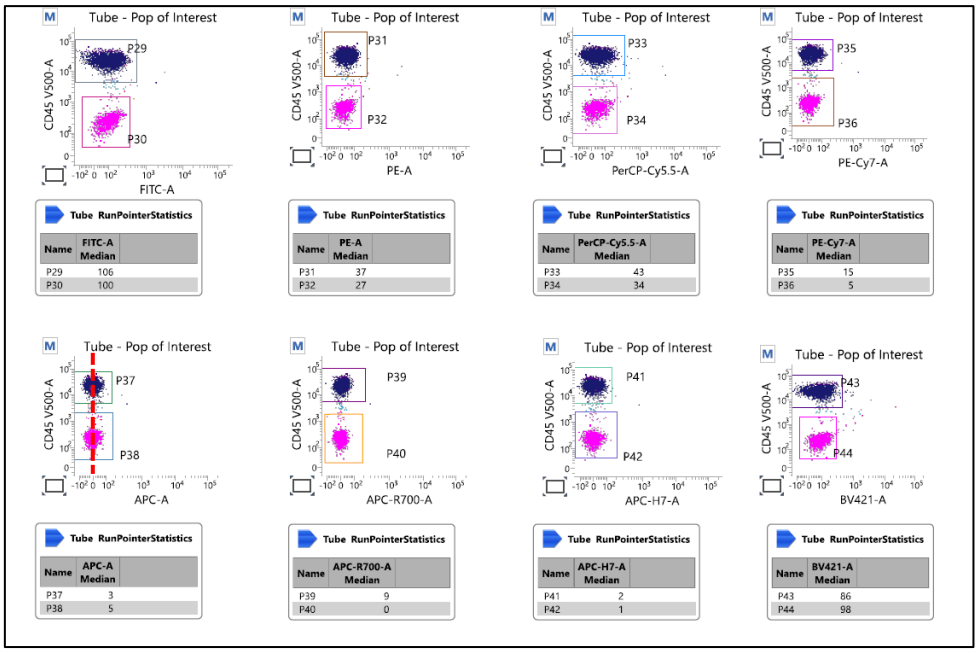

Figure 1: An example worksheet with a bi-exponential scale for checking compensation using the median X-axis values of the positive and negative populations across several fluorescent parameters. Note visual verification line drawn in the V500 vs APC gate and mathematical verification with the MFI in the data boxes.

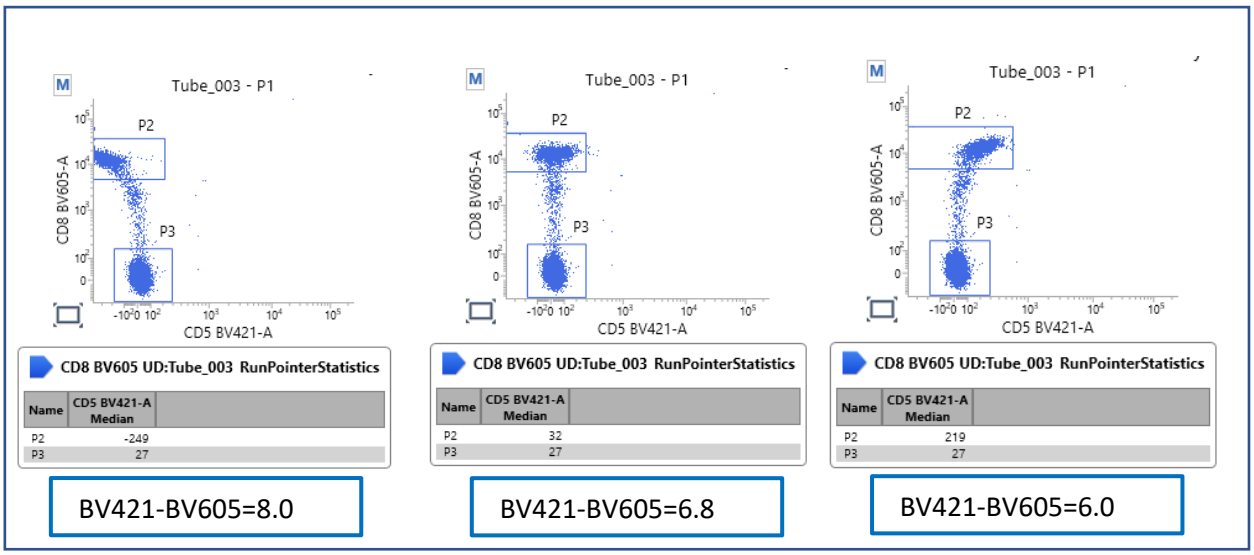

Figure 2: An example worksheet with a bi-exponential scale showing overcompensation (left), proper compensation (middle), and under compensation (right) using the median X-axis values of the positive and negative populations

How is compensation/ Spillover Values (SOV) calculated on the FACSLyric™? (Table 1)

Compensation is calculated in the BD FACSLyric™ FACSuite™ RUO software by taking measurements of the median fluorescence of each channel using the same mathematical equations as the BD FACSDiva™ software. However, unlike other flow cytometers' software programs, this calculation is done a little differently in the FACSuite™ RUO Software. Typically, the user optimizes the PMT voltages for their assay and then compensation is calculated directly from the user's optimized voltages. However, in the BD FACSLyric™ FACSuite™ RUO software, Spillover values (SOVs) are established by incorporating the MFI targets, that are used for the user's optimized voltages, and links them to the MFI targets set by the default reference settings with BD™ CS&T beads. The advantage with this method is that the user will not have to repeat any compensation controls to re-calculate the compensation if the voltages are changed as done with previous versions of BD flow cytometer software. (Exception: this does not apply to *Modified Reference Settings* which will be discussed in later sections).

The BD FACSLyric™ RUO FACSuite™ software uses two default reference Settings created by the manufacturer during installation of the flow cytometer: *Lyse Wash* (LW) and *Lyse No Wash* (LNW). These include preset voltage settings and SOVs. In addition to these two default settings, the user can optimize the default voltages to create their own settings, also known as *Tube Settings*. Tube settings create new MFI targets after running the CS&T beads.

The user can establish their own instrument settings to set Tube Settings and/or Spectral Overlap in three ways:

- Method 1: Lyse Wash or Lyse No Wash reference settings with preset voltages, and the user's own compensation controls to calculate SOV
- Method 2: User Defined (UD) reference settings with user-defined voltages and user-defined compensation controls to calculate SOV
- Method 3: Modified Reference settings with both user-defined voltages AND user-defined compensation controls that allow for editing the calculated SOV

Table 1 - Instrument Setting Methods for the BD FACSLyric™ Using FACSuite™ Software

Term	Synonym	Definition
Lyse No Wash (LNW) reference settings	Preset voltages & SOV	• Voltage settings that place normal lysed, unwashed whole blood lymphocytes on scale • The associated spillover values are measured using single-fluorescence control tubes (BD FC™ beads)
Lyse Wash (LW) reference settings	Preset voltages & SOV	• Voltage settings that place normal lysed, washed whole blood lymphocytes on scale • The associated spillover values are measured using single-fluorescence control tubes (FC beads)
User Defined (UD) tube settings	User's optimized voltages	The user's optimized voltages for each PMT. SOVs are updated after running CS&T™ beads following optimization

User Defined (UD) Reference settings	SOV calculated from user's compensation tubes	Method 1: Default reference setting voltages with user's choice of compensation controls to auto calculate SOVs Method 2: User's optimized voltages and user's choice of compensation controls to auto calculate SOVs. Voltages can be edited (and SOVs will re-calculate automatically if a voltage is changed) but SOV cannot be edited.
Modified Reference Settings	Compensation matrix can be edited	Method 3: User's optimized voltages and user's choice of compensation controls with SOVs that can be edited. Voltages cannot be edited but SOVs can be edited.

BD™ FC beads and Lyse Wash and Lyse No Wash Reference Settings

Lyse Wash (LW) and Lyse No Wash (LNW) Reference Settings are created during initial instrument installation using the BD™ IVD 7 color FC beads, which are surrogates for stained single-fluorescence controls. (**Table 2)** BD™ FC Beads are 3-µm polystyrene beads coupled to fluorochromes and dried down in single-use 12 × 75-mm tubes. Each tube comprises a mixture of positive and negative beads. Additional BD™ FC beads must be purchased by the user because the Reference Settings must be updated every 60 days. The BD™ IVD 7-color FC beads are required for the FACSLyric™ Clinical Software and are used for both the LW and LNW Reference Settings. These Reference Settings can also be used in the FACSLyric™ RUO Software. There are other FC beads for almost every standard BD fluorochrome (**Table 3)** which can be used to build a library of spillover values.

TABLE 2

BD™ FC Beads, 7-Color Kit (CE/IVD)
FITC
PE
PerCP-Cy™5.5
PerCP
PE-Cy™7
APC
APC-Cy7

TABLE 3

BD™ FC Beads (single fluorochromes)
BB 515
APC-H7
APC-R700
V450
V500-C
BV421
BV510
BV605
BV711
BV786

The spillover values created using BD™ FC beads are validated for use with 4- and 6-color IVD-cleared TBNK assays in the FACSuite™ Clinical software. They include both Lyse Wash (LW) and Lyse No Wash (LNW) PMT settings and spillover values that can be used in both the FACSuite™ Clinical Software and FACSuite™ RUO Software. In the FACSuite™ RUO Software, these default settings may not be optimal for some lab developed multi-color panels. However, these settings can be a starting point in assay development and each lab can decide the best method for setting up the compensation matrix of their specific LDT. This method, along with other methods, is described in the steps outlined below.

For any compensation setting in a laboratory developed test (LDT) in the BD™ FACSuite™ RUO software, including Lyse Wash and Lyse No Wash and User Defined settings, FACSLyric™ users have the option to either add new compensation controls (for different fluorochromes not already established with the FC beads) to the default LW and LNW Reference settings or entirely replace the Spillover Values generated using FC bead controls in the reference settings using cells and/or other compensation reference particles as compensation controls (discussed in later sections). Spillover values derived from FC beads are an average of reagents conjugated to that fluorochrome. However, the spillover values of individual reagents may vary from those calculated from the FC beads. Consequently, any new laboratory developed assays should be assessed to determine if the spillover values using FC beads are sufficient or if new compensation controls for that reagent are required. In the authors' experience, FC beads often do not allow for adequate calculation of an optimal compensation matrix in high parameter clinical flow cytometry assays and alternative approaches may be needed.

Choosing different types of single-color controls for setting up Compensation

FACSLyric™ FACSuite™ RUO software allows for the use a wide variety of single-color compensation controls in the same spillover matrix. The user will need to weigh the pros and cons of each one to make an informed decision. Single-stained compensation controls can be prepared from BD™ FC beads, antibody capture beads, stained cells, or a combination of these controls. A single-color control for every fluorochrome in the assay must be run to establish the spillover values. This section will provide an overview of single-color controls and the pros and cons of each one.

1. *BD™ FC beads:*
    - Dried-down polystyrene beads bound to fluorochromes
    - Used on FACSLyric™ to create and update Lyse Wash and Lyse No Wash reference settings every 60 days in the FACSuite™ Clinical and optionally in the FACSuite™ RUO software
    - Reconstitute with bead dilution buffer immediately before use, with a limited time to use after being reconstituted[5]
    - Spillover values represent an average of antibody-fluorochrome conjugates for that reagent
    - Must be used with lot-matched bead files found on the BD website
    - Pros:
        - Require minimal sample preparation time
    - Cons:
        - Spillover values may not be accurate for all reagents
        - Settings expire after 60 days must be repeated in the FACSuite™ Clinical software and in the FACSuite™ RUO software if they are used in that application.
        - Not available for all fluorochromes
        - After rehydration, even when protected from light, the beads have short stability (only 1 hour at 18°C–25°C and only 4 hours at 2°C–8°C)

2. *Antibody Capture beads/ BD™ CompBeads* **(Figure 3)**

- Beads that bind human based cell fluorochrome-labeled antibodies on their surface
- Follow specific manufacturer's protocol for bead preparation[7]
- Can be used when stained cells do not yield enough positive events to calculate compensation or for antibodies that stain in a heterogeneous pattern instead of yielding a tight peak
- Pros:
  - The same antibodies that are used in the panel can be used for compensation
  - The staining process is quick
- Cons:
  - Spillover values may vary when compared to cell staining
  - Cannot be used for calculating compensation for non-antibodies or dye stains such a 7-AAD, Syto™, and FLAER™
  - Might not be best for violet excited dyes where cell autofluorescence is important[9]

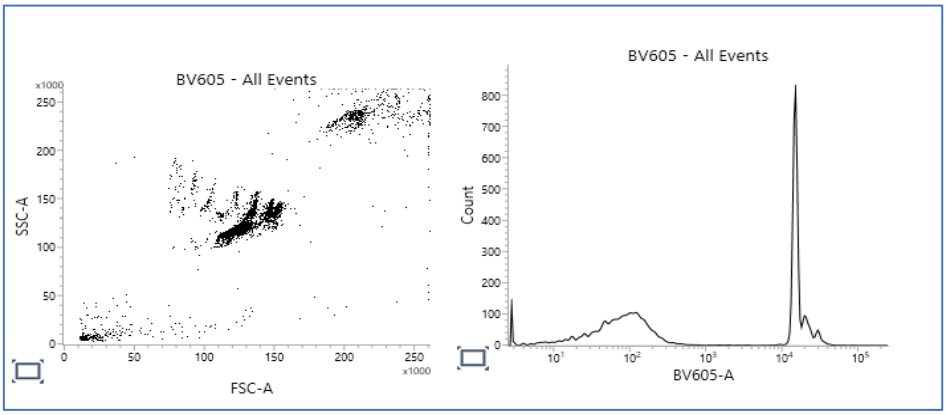

Figure 3: Using BD Comp Beads as a Compensation

3. *Single-stained cells* ***(Figure 4)*:**

- Can be obtained from a fresh specimen or commercially available products
- Should be stained singly with an antibody in every fluorochrome in the panel following the same sample preparation methods used for fully stained samples [for example: adding brilliant stain buffer (BSB) and/or fixative if those are used in the panel]. The BSB product insert states: For the most accurate compensation, compensation controls created with either cells or beads should be exposed to BD Horizon Brilliant Stain Buffer for the same length of time as the corresponding multicolor panel. Additionally, the most accurate compensation will be created when BD Horizon Brilliant Stain Buffer is used in all compensation controls, including Brilliant polymer and non-polymer dyes.[4]
- The sample must include a positive and negative population for the antibody tested in each tube. As each cell population has a unique autofluorescence, it is optimal to assess positive and negative staining on the same cell type[1] (for example, CD8+ cells can be assessed on lymphocytes and CD33+ cells can be assessed on monocytes). There are 3 ways to do this:

- Ensure that there are positive and negative populations in your sample (lymphocytes for CD8+ and CD8-)
- Choose "separate unstained tube" when running the compensation control tubes and use a separate unstained tube for each cell type
- If a negative-stained population is normally not found in the sample, spike the single-color tube with unstained cells for acquisition run of each cell type. (For example, CD45 is positive on all normal WBC's. Add unstained cells to the CD45+ sample just before acquisition of the compensation control).

- The FACSuite™ RUO software can only acquire the same cell type in one acquisition. Multiple acquisition runs may be needed if there are more than one cell type being assessed.
- The positive population should be of similar fluorescence intensity as cells stained with antibodies used in the assay (for example, if using CD4 in every color, for the comp control, the fluorescence intensity must be similar or greater than reagents used in the actual panel)
- Pros:
  - Single-stained cell compensation controls are the closest equivalent to biological samples
  - Inexpensive (no purchase of control specimen)
- Cons:
  - Can be time-consuming to prepare and acquire (only one gated population can be acquired in each single-color control run to account for each cell population's unique autofluorescence). For example lymphocyte-gated antibodies should be acquired and then monocyte-gated antibodies should be acquired separately
  - The sample may not include enough positive events (for example CD34+) to calculate the compensation.
  - Might not work effectively with "stacked" antibody combinations i.e. CD8/ Lambda FITC

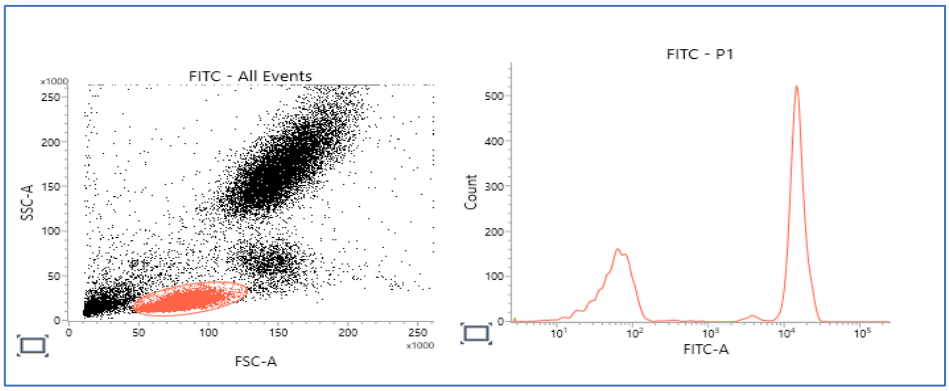

Figure 4: Using Stained Cells as a Compensation control

Each lab will need to evaluate if bead-based or cell-based compensation is adequate. In some cases, the calculated compensation may be over- or under-compensated with one or the other comp materials. See **Figure 5.**

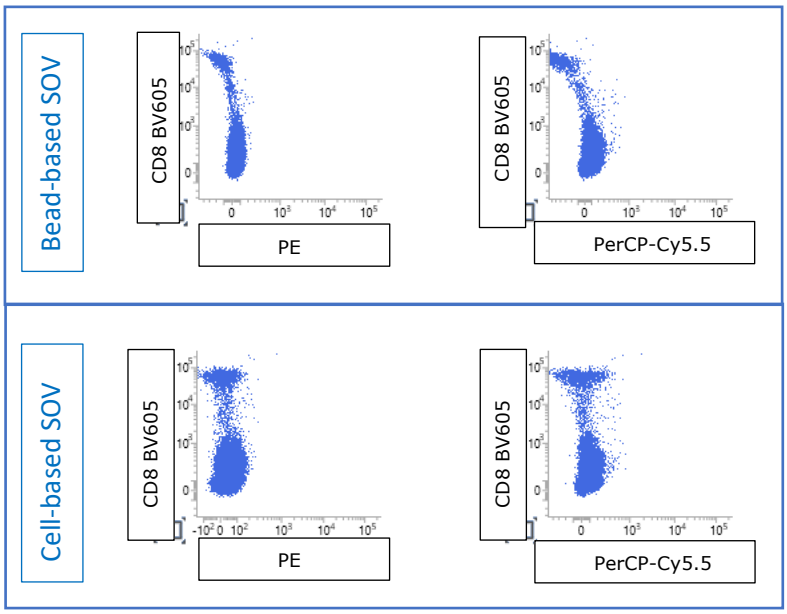

Figure 5: Using BD Antibody Capture Beads vs Stained cells for Compensation controls – top line displaying CD8 BV605 stained cells using **bead**-based SOVs, bottom line displaying CD8 BV605 stained cells from **cell**-based SOVs. In this case, SOV generated using stained cells provided a better SOV. Each laboratory should verify a suitable method for creating SOVs.

The user has the option to use any single-color control (cells, antibody capture beads, FC beads or combination of these) for any fluorochrome. Follow the procedure in the FACSuite™ RUO Reference manual for adding new fluorochromes to the software Library. 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.[1] Tandem dyes also tend to be more susceptible to degradation due to light, temperature or fixation which over time or under different experimental conditions can cause changes to the fluorescence which may now be over- or under-compensated using the SOVs previously established for that fluorochrome. These potential variations can be controlled by re-assessing spillover values with the current lot/open vial of reagents. Because the BD FACSLyric™ may be very sensitive to slight differences in tandem dye variations different lot-numbers of the same reagent can have different spectral properties. It is recommended that tandem dyes may benefit from tandem specific or tandem lot-specific compensation. This may require tandem-specific or even tandem lot-specific single-color controls be set up with cells and/or antibody capture beads depending on the unique needs of the laboratory's panels. As more tandem dyes become available, understanding each dye's unique chemistry can inform users on whether lot-specific compensation is necessary

BD FACSLyric™ FACSuite™ RUO panel design considerations

Setting up a new assay requires multiple steps that may require some iteration. Although this module will not discuss panel design, it is important to consider the density of the target antigens, the brightness of the fluorochrome and the spread of the fluorochrome into other channels when matching antibodies and fluorochromes.[6] Once a panel is designed, the instrument settings should be optimized (i.e. set the voltages or verify that the default settings are valid for all parameters including light scatter and fluorescence) to ensure that all populations are on-scale.[2]

Lyse Wash/ Lyse No Wash Reference Settings components:

As the names imply, the Lyse Wash (LW) Reference Settings are optimized for assays with wash steps while Lyse No Wash (LNW) Reference Settings are optimized for assays with no wash steps. Both of these Reference Settings contain two components that are important for compensation setup. The first component is "Tube Settings" which includes voltages that are specific to the instrument and are directly

related to the specific instrument's Mean Fluorescent Intensity (MFI) targets set by the tube target values (TTVs). The other component is measured "Spillover Values" or SOVs.

Tube Settings standardize assay performance daily when CS&T beads are run during Performance QC (PQC). The Tube Settings include MFI targets for voltage settings, threshold, flow rate, window extension, and the area scaling factor. Since Tube Settings are calculated using CS&T beads, it is critical that users upload the correct CS&T bead lot file to match the bead lot that is currently in use. CS&T beads **should be used within 20 minutes after reconstitution and protected from light** to minimize performance issues from possible light degradation.[3] When running CS&T beads during Performance QC and subsequently in Assay/Tube Settings Setup, the PMTs are adjusted to reproducibly hit target values so that assay performance is consistent from day to day.

In addition to Tube Settings, the Reference Settings also contain Spillover Values (SOVs) for compensation. For Lyse Wash and Lyse No Wash settings, the SOVs are calculated using FC beads and must be updated every 60 days. The Lyse Wash and Lyse No Wash Reference Settings may be an easy starting point for assay development for laboratory developed tests but should be verified to be appropriate according to regulatory guidelines. The default settings may not be optimal for some lab developed multi-color panels. Other techniques that may be useful for setting up compensation on the 12-Color BD FACSLyric™ using FACSuite™ RUO software are discussed below.

Setting up User-Defined Tube and/or Reference settings:

If Lyse Wash or Lyse No Wash Reference Settings do not produce adequately compensated results for any given assay, another method would be to establish *User Defined* (UD) settings in the FACSuite™ RUO software. All newly created Tube and Reference Settings are saved in the FACSuite™ RUO Library and can be used as needed to build assays. The following steps discuss basic principles on how to optimize the voltages for the assay along with three methods that may be used to establish compensation.

Creating optimized voltages (Tube Settings, Tube Target Values TTVs, MFI target values)

The default Reference Settings (containing voltages needed to reach preset Tube Target values using BD™ CS&T beads) may work for most reagents and starting with the default settings might reduce the number of adjustments needed to get the cell populations on scale. However, each channel should be evaluated for voltage optimization. For each detector, the user needs to find the optimal voltage/MFI target that will keep the bright signals in the linear range of the detector (on scale) and optimize the stain index (normalized signal:noise ratio). By running single stained cells, the user can adjust the voltages in Preview mode, to find the optimal voltage setting for each PMT. See **Figures 6 and 7.** After optimizing the MFI targets are then updated by creating new tube settings by running BD™CS&T beads.[(2)]

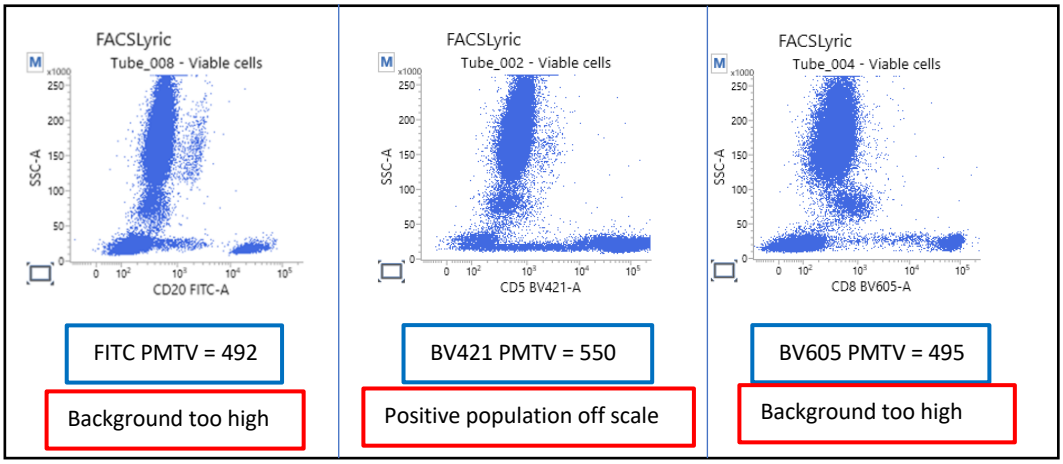

Figure 6: Using the default Lyse Wash PMTV (Photomultiplier Tube Voltage) settings, the negative and positive populations shown for FITC, BV421, and BV605 are not ideal for the LDT and require optimization.

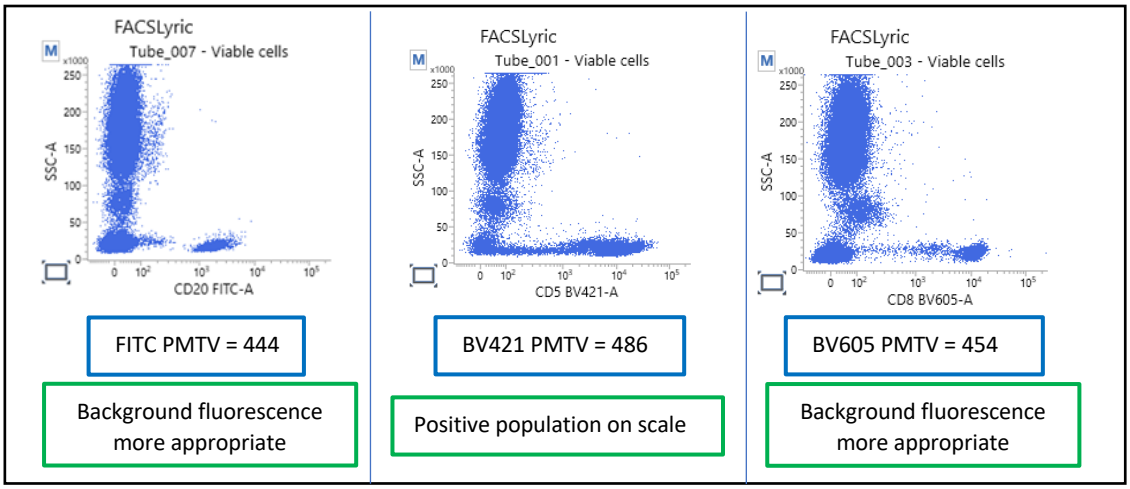

Figure 7: Optimized settings after adjusting the PMT voltages to appropriately position the negative and positive cells for the associated LDT.

a. Verify that the compensation on the fully stained sample is acceptable. Once verified, use the data to create new Tube Settings using BD™ CS&T beads to reflect these voltage changes and generate new MFI targets. This will create new Tube Target Values (TTVs) that will include the re-calculated SOVs from the LW or LNW reference settings.

b. If the compensation on the fully stained tube is not acceptable (see **Figure 8**), the user will need to consider alternative methods to optimize spillover values. See steps below for some options.

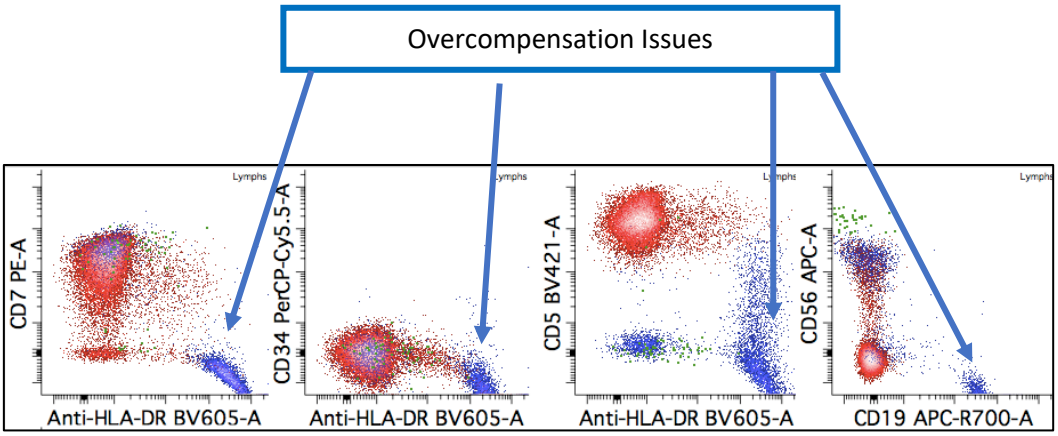

Figure 8: Default LW Reference Settings may sometimes demonstrate compensation errors such as this overcompensation of the x-axis signal vs the y-axis signal.

Optional Methods to create Spectral Overlap Values (SOVs)

In some assays, using default SOVs contained in the LW or LNW Reference Settings may not be suitable. In these cases, the user has three alternative methods that can be used to correct this in BD FACSuite™ RUO software. Spillover values may also be modified as appropriate using post-acquisition software.

Methods for SOV optimization **(Table 4):**

(1) Create tube specific User defined reference settings (for one tube only)
(2) Create new User defined reference settings to entirely replace/overwrite the SOV generated by the default LW reference settings (for all tubes)
(3) Adjust/edit SOVs and save as modified reference settings

Table 4 Options for Setting Up User-defined Reference Setting SOVs

Method	Definition	Pros	Cons
(1) User Defined	SOV for one tube	The user can choose their own compensation controls for one tube that does not have appropriate compensation with default SOVs	Only for one tube
(2) Replace LW Reference settings	SOV for every tube	Complete compensation matrix for all assays	Can be cumbersome to set-up
(3) Modified Reference Settings	SOV that can be edited	SOV settings can be edited at any time	Voltages cannot be edited at any time

CAUTION: the next steps can be complicated and lengthy. The user is strongly encouraged to contact a BD technical application scientist for guidance and instruction before making new User Defined Reference Settings or Modified Reference Settings.

Method 1 - Using user's optimized voltages and creating *User Defined (UD) Reference Settings* for **one tube**

New User Defined Reference Settings (the user's voltages with an alternate method for compensation calculation) can be created to replace the BD Lyse Wash or Lyse No Wash Reference Settings for **a single tube**. This will generate both new tube settings (with optimized voltages) and new spillover values (SOVs) for the one tube. Unlike Lyse Wash and Lyse No Wash reference settings, these UD Reference Settings are updated through the Assay/Tube Settings Setup (ATSS) function after Performance Quality Control (PQC) using BD™CS&T beads is

completed. Assay/Tube Settings Setup (ATSS) calculates updated SOVs using CS&T beads to adjust for daily instrument performance fluctuations.

Advantages:

- the user can apply different or same reference settings for each tube in one assay (for tandem-specific needs or lot-specific needs, etc.) while having the rest of the tubes linked to the default LW Reference Settings
- will not require an update after 60 days therefore SOV re-calculation frequency will be at the discretion of the user

Disadvantage:

- UD Reference Settings will have to be updated separately from LW and LNW Reference Settings when the SOV needs to be recalculated.

Procedure:

a. On the tube that needs UD Reference Settings, right click and choose "Create Reference Settings" and name as appropriate and add description if needed. Newly created UD Reference Settings will be applied to this tube only however the user can apply this newly created Reference Settings to subsequent tubes as needed if the fluorochromes are exactly the same.
b. The "Add Fluorochrome" function will prompt the user to define the single-color fluorochrome controls (FC beads, capture beads, biologically relevant cells, or a combination of controls) to calculate spillover values to generate an updated compensation matrix.
c. Follow the software prompts to select the appropriate lot of CS&T beads and acquire the beads followed by the single-color compensation controls.
d. Gate appropriately and Finish.
e. Run a fully stained tube and verify that the compensation is acceptable. Note: each laboratory should establish their own criteria for acceptability.
f. If all compensation is acceptable, right-click and "clear tube".
g. Create/Overwrite or Save the Assay.
h. If this tube is used in other assays, the newly created UD Reference settings will have to be selected in the Tube Settings and the user will have to Create/Overwrite that assay.
i. If the compensation on the fully stained tube is not appropriate, consult the "Practical Tips" below and/or continue to Method 3.

Method 2 - User's optimized voltages and *User Defined Reference Settings* for **all tubes**

New LW Reference Settings can be created to entirely replace the BD Lyse Wash Reference Settings for **all tubes**.

Advantages:

- Uses new optimized tube settings and creates spillover values (SOVs) for all tubes with the user's compensation controls
- Does not require an update after 60 days. SOV re-calculation frequency is at the discretion of the user

Procedure:

a. In the *Library* under Tube Settings, choose BD, then choose Lyse Wash and click the Spillover Values tab.
b. Click "Remove" and select all fluorochromes except one (for example, do not remove FITC).
c. Under the "Setup and QC" tab, choose "Add fluorochromes" from the Task menu.
d. Add the fluorochromes removed in the previous step with appropriate control type (except the non-deleted one) to the Lyse Wash Reference Settings.
e. Acquire compensation controls for each fluorochrome added. Gate if necessary and "Finish".
f. In the *Library* under Tube settings, choose BD, choose Lyse Wash and click the Spillover Values tab.
g. Repeat step b to remove/delete FITC (or the one that was not deleted in step b) and repeat steps c-e to add the non-deleted fluorochrome to the Reference Settings.
h. The Expiration date in the Library for Lyse Wash should now say N/A.
i. Run a stained sample using the new Reference settings and verify that the compensation on the fully stained sample isappropriate. If so, Create/Overwrite or Save the Assay.
j. If the populations are not appropriately compensated consult the "Practical Tips" and/or continue to Method 3.

Practical tips when using compensation controls to auto-calculate SOV

Sometimes the initial attempt at running compensation controls to auto-calculate SOVs may not be successful. If so, several options may be considered to optimize spillover values:

- Remove the problematic reagent/mAb from the Lyse Wash library and then add it back with an alternate reagent. Stain the cells with a different antibody but with the same fluorochrome. A good option is CD8 which can be substituted/used in the fluorochrome of interest because it is expressed at a high level and the lymphocyte population typically includes both CD8 negative and CD8 positive staining populations.
- Pre-washing the blood sample prior to staining helps eliminate serum proteins that might be causing a higher background signal due to non-specific binding of reagents.
- Include a separate unstained tube.
- Verify populations of interest are gated appropriately for each acquisition run to allow for cell's unique autofluorescence (i.e. monocytes are run separately and gated for CD33)[1]
- Verify that the panel does not include multiple IgG2 antibodies that could be providing unfavorable interactions [8]
- For any tube/ panel that includes BV dyes (BV421, BV510, etc.) verify that Brilliant Stain Buffer (BSB) has been added if it is used in the cocktail (even for a single color control)[4]

Method 3 - Modified Reference Settings allow the user to adjust/edit the SOV of previously created UD Reference Settings for **one tube only**. This is the **only** method that allows the user to adjust the compensation and save it as a new (modified) setting.

Modified Reference Settings (using the user's optimized voltages with the user's method for compensation controls) can be created to replace the BD Lyse Wash (LW) or Lyse No Wash (LNW) Reference Settings for **a single tube**. This will generate both new tube settings (with optimized voltages) and new spillover values (SOVs) that can be edited for the one tube. Unlike Lyse Wash and Lyse No Wash reference settings, these Modified Reference Settings are updated through the Assay/Tube Settings Setup function after PQC is completed. Assay/Tube Settings Setup (ATSS) calculates SOVs using CS&T beads to adjust for daily instrument performance fluctuations.

Principle: Modified reference settings **for one tube** can be created by editing previously created User Defined Reference Setting compensation and saving them as *Modified Reference Settings*.

Advantage: the user can edit/adjust the compensation values as needed which might be observed with new lot variability or cocktail dye-dye interactions that may happen over time.

Disadvantage: **Only** the SOV can be adjusted/edited. If the Tube Settings (PMT voltage) needs to be adjusted, the UD Reference Settings will have to be created again (Method 1) and then edited and saved as the Modified Reference Setting.

Procedure:

a. The correctly adjusted compensation values should already be established using a fully stained tube.
b. The *Library* should include each antibody-fluorochrome conjugate listed in Reagents including the Lot ID. For example, under Lot ID, one could enter "SPECIFIC" with an expiration date of 1/1/3000 (or enter the exact manufacturer's lot information).
c. In *Experiment*, choose "New From Assay", open a previously created Assay, open *Tube Properties* and under the *Parameters* tab, change one of the volts by a decimal.
d. Under *Reagents*, confirm that the correct fluorochrome and antibody are listed. For the Lot ID, choose the previously entered name "SPECIFIC" or the appropriate lot number.
e. Right click on the tube and choose "Create Reference Settings". This opens a new window that allows the user to add the new fluorochromes previously added to the Library (step b).
f. Follow FACSuite™ RUO Software prompts to select appropriate lot of CS&T beads and define the single-color controls (FC beads, capture beads, biologically relevant cells, or a combination of controls) to calculate spillover values to generate an updated compensation matrix. Add fluorochromes for all the ones that required adjustment of the compensation established in step (a). Alternately, the user can add in all fluorochromes and this will ensure that any SOV value can be adjusted as needed in the future.
g. Choose the appropriate "SPECIFIC" lot or manufacturer lot number under LOT ID.
h. Acquire the single-color compensation controls, gate, Finish. Note: if using stained comp beads gating will be automatically applied.
i. Save the new Reference Settings with a unique name.
j. Run a fully stained sample and edit the compensation values as needed then right click on the tube and select "Save Modified Reference Settings" and name appropriately.
k. Click "Save Assay/Overwrite" to apply these changes for the Assay.
l. Optional: The earlier version of the reference settings can be deleted in the Library while keeping the Modified Reference Setting.
m. Periodic verification of the compensation on a fully stained sample at a user-defined interval is recommended. **(Figure 9)** If the compensation of the fully stained tube requires adjusting, the Modified Reference Settings can be edited and saved.

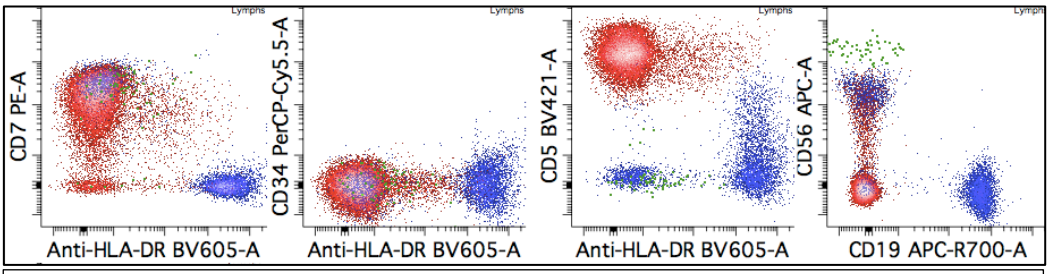

Figure 9: Modified Reference Settings with appropriately compensated data

Frequency for Updating Compensation…and the process

The frequency of updating the Spectral Overlap values (SOVs) is laboratory-dependent. In the case of LW and LNW Reference settings, FACSuite™ Software requires performing an update every 60 days if FC beads are used. In the case of Lab Developed Tests with customized Tube Settings or UD Reference settings/Modified Reference Settings, the laboratory can determine the frequency of the updates. It can be a regularly scheduled event (such as every 2 weeks, every 2 months, etc.) or when something changes in the optical pathway (i.e. new laser, new filters, major maintenance).

Suggested steps to re-establish Spectral Overlap Values

1. Run CQC (Characterization Quality Control) using BD™ CS&T beads
2. Run ATSS (Assay/Tube Settings Setup) which adjusts the voltages to place the BD ™CS&T beads to the previously established MFI targets
3. Update all Reference Settings used in FACSuite™ RUO (LNW, LW and any User-Defined (UD) or Modified Reference Settings)- this step could be quite lengthy depending on your process.
4. Acquire a fully stained tube and verify that the results demonstrate acceptable compensation.

Quality control of spillover values and compensation matrices

Compensation settings must always be assessed for each assay. It is recommended to monitor compensation periodically. The method and frequency are up to each laboratory. One potential way to do this, is to create bi-exponential 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.

Additional Points to Remember

- Run CS&T beads within 20 minutes after preparation for accurate QC and settings updates [3].
- Reconstituted FC beads should not be exposed to light or RT for more than 1h at RT (or 4h at 4°C).[5]
- Preparing single color controls - The controls should be prepared with the identical sample preparation as the fully stained samples (i.e. Brilliant stain buffer (BSB), fixation, etc.) [4].
- If two or more Brilliant Violet (BV) dyes are in the same tube, Brilliant Stain Buffer should be added to the tube before fluorochromes are added.

- Validate cocktail stability over time - The product insert for BD™ Brilliant Stain Buffer indicates that a cocktail with BV dyes is only good for 24h in a fully stained tube, therefore the LDT cocktail needs to be validated for stability over time.[4] When cocktails are stored beyond 24h, Brilliant Violet dye-dye interactions will increase, potentially affecting the compensation.[6] The laboratory needs to determine the shelf-life of each cocktail. See **Figures 10-13.** It is noted that, these dye-dye interactions may be mitigated by adjusting SOVs by editing Modified Reference Settings or by applying post-acquisition compensation as illustrated in Figure 12 and 13; however, such adjustments should be undertaken with extreme caution.

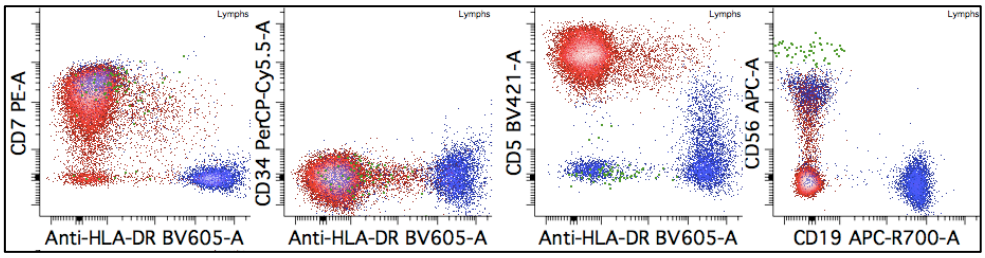

Figure 10: Cocktail – Day 0 (fresh cocktail) – using Modified Reference Settings

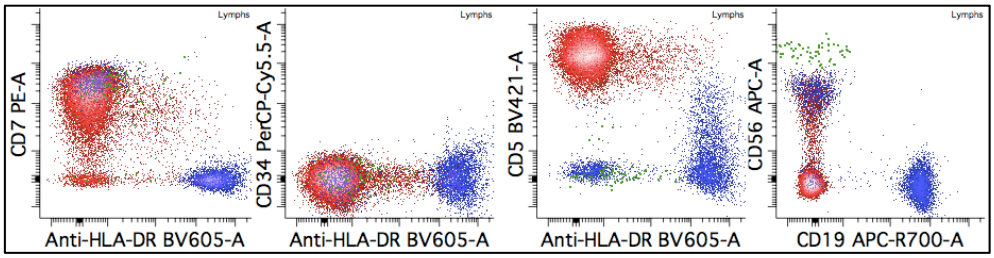

Figure 11: Cocktail – Day 1 (same Modified Reference Settings), showing little or no changes.

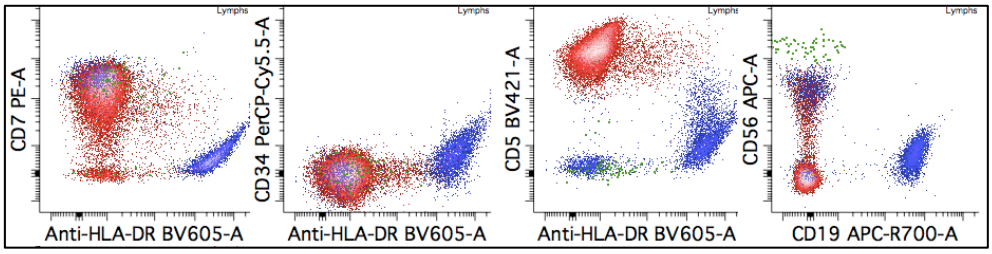

Figure 12: Cocktail – Day 7, using same Modified Reference Settings, showing multiple compensation errors.

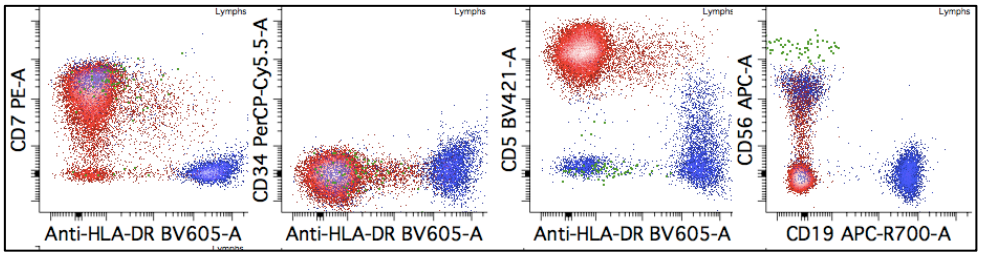

Figure 13: Cocktail – Day 7, after updating/editing Modified Reference Settings

Conclusion:

Newer flow cytometers have many features that automate the daily quality control measurements and standardize instrument setup. These advances in technology ensure high quality results. However, with the increasing number of fluorochromes and detectors used on these new platforms, the default settings may not be optimal for some lab developed multi-color panels. This document describes some of those challenges along with steps that may be useful for setting up compensation on the 12-Color BD FACSLyric™ using FACSuite™ RUO software. The reader is encouraged to contact the technical support team at BD for more detailed instructions on creating and applying user-defined settings.

References:

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3. BD™ CS&T Research Beads product page: https://www.bdbiosciences.com/content/dam/bdb/processed-assets/documents/23-11519.pdf
4. BD™ Brilliant Stain Buffer product page: https://www.bdbiosciences.com/en-us/products/reagents/flow-cytometry-reagents/research-reagents/buffers-and-supporting-reagents-ruo/brilliant-stain-buffer.563794
5. BD™ FC Beads product page: https://www.bdbiosciences.com/content/dam/bdb/products/global/reagents/flow-cytometry-reagents/clinical-discovery-research/controls-and-supporting-reagents-ruo-gmp/661631_base/pdf/23-19069.pdf
6. BD Webinar "Enhancing the Performance of BD Horizon™ Brilliant Violet™ Reagents": https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=&cad=rja&uact=8&ved=2ahUKEwjZrtq_rZz4AhWln4QIHZeeAU8QFnoECAgQAQ&url=https%3A%2F%2Fwww.bdbiosciences.com%2Fcontent%2Fdam%2Fbdb%2Fmarketing-documents%2FEnhancing%2520the%2520Performance%2520of%2520BD%2520Horizon%25E2%2584%25A2%2520Brilliant%2520Violet%25E2%2584%25A2%2520Reagents_Multicolor-Webinar-2013-11-BV-Reagents.pdf&usg=AOvVaw26P8KusOjmaLTEeVjrNGgV
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Glossary:

BD CS&T™ Beads: Particles that are hard dyed at three different intensities to emit in a broad spectrum that is detectable by all channels of the BD FACSLyric™ Flow Cytometer. BD CS&T Beads are used with the software to automatically set up tube settings, assays, and characterize and track cytometer performance.

BD FC™ Beads: Single-color Particles that are used to create or update reference settings for BD standard fluorochromes (see table x for BD standard fluorochromes). Additional beads are available as RUO

Characterization QC (CQC) (Also known as "Baseline" on other BD flow cytometers): The process that establishes target cytometer settings and sets pass or fail values for linearity, sensitivity and %rCV.

Compensation: The mathematical process by which the fluorescence values for the primary detector reflect only the fluorescence of the intended fluorochrome and the fluorescence from any other fluorochromes is removed.

Lyse Wash (LW) reference settings: Factory pre-set 'reference settings' which contains MFI targets (used to set the PMT voltages) and spillover values (SOV) for specimens that are lysed and washed. The targets are cytometer settings that place normal lysed whole blood lymphocytes on scale for FSC, SSC and fluorescence parameters using an FSC threshold. From these MFI, the SOVs are generated with FC beads.

Lyse No Wash (LNW) reference settings: Factory pre-set 'reference settings' which contains MFI targets (used to set the PMT voltages) and spillover values (SOV) for specimens that are lysed and not washed. The targets are cytometer settings that place normal lysed, unwashed whole blood lymphocytes on scale for FSC, SSC and fluorescence parameters using a PerCP or PerCP-Cy5.5 threshold.

Median fluorescence intensity (MFI): The fluorescence intensity value of the event in a defined population that has an equal number of events with fluorescence intensities higher and lower than it.

Performance QC (PQC): The set of automated software functions used to measure and track cytometer operation, and to set up consistent Lyse Wash and Lyse No Wash tube settings.

Reference settings: Tube settings and the associated spillover values that have been measured using fluorescence control tubes.

Spectral overlap: Overlap of the emission spectra of two fluorochromes.

Spillover: For a given fluorochrome, fluorescence detected in a secondary detector.

Spillover Value (SOV): The ratio of the delta MFI (positive population - negative population) of the fluorescence in a secondary (i.e., spillover) detector divided by the delta MFI of the primary detector. SOVs are the mathematical factors used in the compensation process.

Stain index: The resolution of the two populations depends on the difference between the positive peak mean and negative peak mean and the width of the background peak.

Threshold: A trigger signal and level of discrimination to eliminate unwanted events. Only events with parameter values above the threshold will be recorded.

Tube settings: A collection of values that place the population at the same position (brightness) whenever the tube settings are applied. Tube settings allow the system to produce comparable results from day to day and from system to system.

Tube Target Values: Tube Settings include mean fluorescence intensity (MFI) targets for voltage settings, threshold, flow rate, window extension, and the area scaling factor.

Window extension: The time added to the pulse duration above the threshold to give the total time during which a pulse is sampled.

Worksheet: An area within the software where plots, gates, statistics, and other elements are created and modified.