Assay Portability on the BD FACSVerse™ System

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Application Note

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Summary

As a result of advancements in technology, different laboratories across the globe are performing collaborative research to decipher the complexities of the human immune system and to monitor diseases. To conduct collaborative and longitudinal flow cytometry based studies, standardization of flow cytometry instruments is essential for obtaining consistent and reproducible results. In addition, flow cytometry assays need to be reusable and transportable for such studies. The BD FACSVerse™ system addresses these needs and provides comprehensive tools for easy, single-step instrument setup. The assays in the BD FACSVerse system include acquisition settings, analysis plots, gates, and statistics, which are reusable on the same system on different days for longitudinal studies and are transportable across different BD FACSVerse systems for multisite studies. Moreover, the spillover values (SOVs) for standard fluorochromes reside in the system and can be automatically applied, which eliminates the need to run compensation controls every day.

In this application note, we have demonstrated the reproducibility of data and portability of assays on the BD FACSVerse system using a T-memory effector panel. Samples from two donors run on different days (over a period of 25 days) showed less than 10% variability when the median fluorescence intensities (MFIs) of the positive populations of different markers were compared. When the same samples were acquired on three different BD FACSVerse instruments by different operators with different levels of experience, using the same assay, the data revealed minimal variation in MFI values for most of the markers in the panel. In addition, SOVs from day 1 of the study were reapplied on subsequent days, which produced accurate compensation over time. Finally, to show reproducibility of instrument setup and assays, the populations identified and gated on day 1 on instrument 1 were reproducibly identified on subsequent days and on different instruments, without any need for adjustment of gates.

BD

BD FACSVerse Assay
A collection of instructions for BD FACSuite software that includes tube settings, an acquisition worksheet, and an analysis report with plots, gates, statistics, and expressions. Assays are reusable and are deployable across different BD FACSVerse systems. The two types of assays are BD-defined and user-defined assays.

MFI Target Values
For given parameters in an assay, the median fluorescence intensities at which the PMTVs are optimal for adequate separation of dim versus bright signals.

Tube Settings
Include a collection of attributes that are used to place the positive population at the same position (MFI target) on the fluorescent scale whenever the tube settings are applied to tubes. Tube settings allow the system to produce comparable results from day to day and from system to system.

Spillover Values (SOVs)
The amount of spectral overlap calculated as the ratio of the primary signal in the channel to any interfering signal from another channel.

Performance Quality Control (QC)
The set of automated software functions used to measure and track cytometer operation and to set up consistent BD-defined (LW and LNW) tube settings. This process:

1. Reduces daily setup time,
2. Enables assays and experiments to be used on different systems and in different labs and produce equivalent results,
3. Sets initial PMTVs and SOVs for BD-defined assays and any user-defined assays,
4. Provides the ability to add SOVs for a single fluorochrome without having to run the other fluorochromes again.

Introduction

Flow cytometry is an essential tool used in research laboratories for performing multiparametric single cell analysis. Monitoring the immune system is a widely used application during which different subsets of immune cells such as lymphocytes, monocytes, and granulocytes are identified by surface markers using multicolor flow cytometry. These immunophenotyping assays often are used for longitudinal studies within a laboratory and for collaborative studies in different laboratories across the world.[1] To characterize the immune phenotypes in such longitudinal and multisite studies, the immunophenotyping assays must be standardized and must be able to be transported and reused consistently and reproducibly. This standardization helps researchers rule out any instrument- or operator-related variation in the results.

In a flow cytometry based assay, there are a variety of steps that need to be optimized and standardized to achieve consistent results, especially in longitudinal and multisite studies. Of all the variables for effective standardization, the instrument setup is the essential component. Moreover, in multicolor flow cytometry, the spillover must be corrected with proper compensation setup by using compensation controls. Flow cytometry users frequently ask the following instrument setup and standardization questions:

- Is there an easy and reproducible way to set up the instrument and assays for day-to-day use?
- Are there any day-to-day variations related to the instrument setup that could contribute to variation in the results over a period of time?
- Are the results comparable among different laboratories (geographically separated) running similar assays on different instruments?
- Is it necessary to run compensation controls with every experiment?

The BD FACSVerse system is a high performance flow cytometer that uses BD FACSuite™ software for acquisition and analysis and addresses all these questions. The system incorporates easy-to-use setup and provides assay portability, thereby making standardization across different instruments very straightforward. The BD FACSVerse assays include tube settings and acquisition and analysis worksheets with plots, gates, and statistics. These assays can easily be reused and transferred to other BD FACSVerse systems. The setup procedure involves single-step daily Performance QC using BD FACSuite™ CS&T research beads to measure and adjust cytometer photomultiplier tube voltages (PMTVs). This ensures that the MFI target values of the assays are maintained without any day-to-day variations. These target values also can be easily exported to other instruments where Performance QC can automatically adjust the cytometer's PMTVs to maintain the target MFI of the assay on each new instrument. Overall, the assays, combined with Performance QC, allow the system to produce comparable results from day to day and from system to system.

The BD FACSVerse system comes with two default tube settings, lyse/wash (LW) and lyse/no-wash (LNW), which are adequate for most human hematopoietic cell types and work optimally with standard fluorochromes. If the default LW and LNW settings are not optimal for a cell type or application, such as intracellular staining, the PMTVs can be optimized for the cells of interest. The optimized user-defined tube settings can be saved and reproducibly used from day to day on a given instrument and can also be exported to other instruments. The SOVs required for compensation reside in the system and can be automatically applied and reused for 30 days without the need to run compensation controls. For assays involving tandem fluorochromes, for which lot-specific compensation is needed, SOVs can be added for a particular fluorochrome without having to run all the fluorochromes in the panel again. Figure 1 summarizes the BD FACSVerse features and the benefit of using the assays.

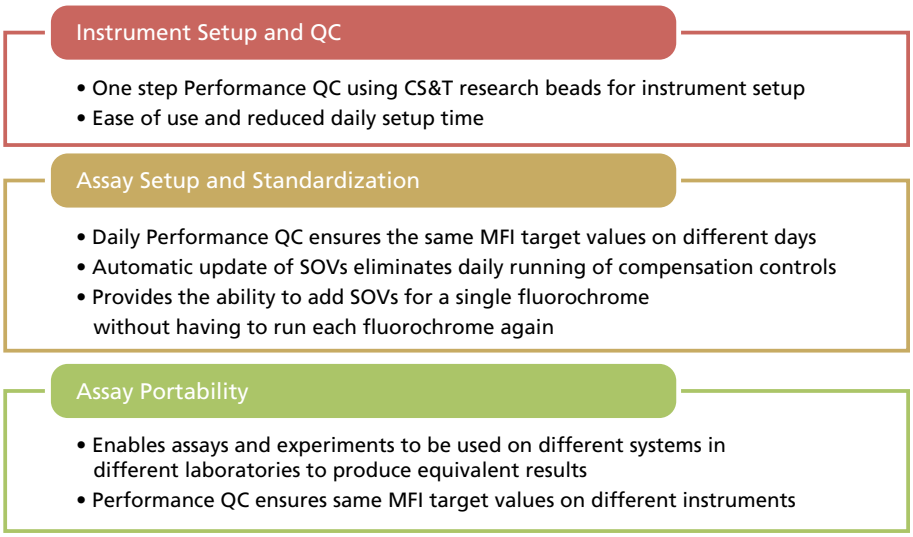

Figure 1. Overview of BD FACSVerse features.

Overall, the Performance QC on a BD FACSVerse system is a very easy to use single-step procedure that ensures that any stored assays and tube settings can be reused to generate reproducible results over time and on different systems run by different operators. To highlight the ease of use, portability, and reproducibility of assays on a BD FACSVerse system, we have used an example of a 7-color T-memory effector panel consisting of CD45RA FITC, CCR-7 PE, CD8 PerCP-Cy™5.5, CD4 PE-Cy™7, CD27 APC, CD3 V450, and CD45 V500 markers.

Objective

The objective of this application note is to demonstrate the ease of use of the BD FACSVerse instrument setup along with standardization and portability of assays. A proof of principle experiment from a T-memory effector assay is presented to show consistency and reproducibility of data collected on:

- Three different days (1, 7, and 25)
- Three different instruments and different operators

Methods

Antibodies

Antibody Specificity	Clone	Isotype	Fluorochrome	BD Cat. No.
CD45RA	L48	Ms IgG1, κ	FITC	347723
CCR-7	150503	Ms IgG2a	PE	560765
CD8	SK1	Ms IgG1, κ	PerCP-Cy5.5	341051
CD4	SK3	Ms IgG1, κ	PE-Cy7	348789
CD27	L128	Ms IgG1, κ	APC	337169
CD3	UCHT1	Ms IgG1, κ	BD Horizon™ V450	560365
CD45	HI30	Ms IgG1, κ	BD Horizon™ V500	560777

Reagents and Materials

Product Description	BD Cat. No.
BD FACSuite CS&T research beads (CS&T research beads)	650621
BD Falcon™ 15-mL conical tubes	352097
Stain Buffer (BSA)	554657
BD™ CompBead Compensation Particles Anti-mouse Ig, κ (BD CompBeads)	552843
BD FACS™ lysing solution (10X)	349202
Polystyrene tubes (12 x 75 mm)	352052

BD FACSVerse Instrument Configuration

Three BD FACSVerse instruments with following configuration were used in the study.

Wavelength (nm)	Detector	Dichroic Mirror (nm)	Bandpass Filter (nm)	Standard Fluorochrome
488	A	752LP	783/56	PE-Cy7
	B	605LP	700/54	PerCP-Cy5.5
	D	560LP	586/42	PE
	E	507LP	527/32	FITC
640	A	752LP	783/56	APC-H7
	B	660/10	660/10	APC
405	A	500LP	528/45	BD Horizon V500
	B	448/45	448/45	BD Horizon V450

Specimens

Blood specimens were collected from normal healthy donors who consented to participate in an Institutional Review Board-approved protocol.

Sample Preparation

1. A seven-color cocktail was prepared fresh by mixing all the antibodies listed in the preceding table using the recommended volume per test provided in the technical data sheet (TDS) for each antibody.
2. Whole blood (100 µL) from each donor was added to the mixed cocktail in polystyrene tubes. The tubes were vortexed gently and incubated for 25 minutes at room temperature (RT) in the dark.

3. Two milliliters of 1X BD FACS lysing solution was added to the each tube, followed by vortexing thoroughly. The tubes were incubated for 10 minutes, at RT, in the dark.
4. The tubes were centrifuged (500*g*, 5 minutes, RT), and cell pellets were washed with 2 mL of PBS.
5. The cell pellet from each tube finally was suspended in 0.4 mL of Stain Buffer. Tubes were vortexed and stored at 2–4°C in the dark until acquisition within 4 hours.

Preparation of BD CompBeads for PE-Cy7 Lot-Specific Compensation

1. One drop of negative BD CompBeads and one drop of anti-kappa BD CompBeads were mixed in a polystyrene tube.
2. PE-Cy7 antibody (from the same lot used to stain samples) was added to the tube using the recommended test volume provided in the TDS and incubated for 15–30 minutes, at RT, in the dark.
3. Two milliliters of Stain Buffer was then added. The tube was vortexed and centrifuged (500*g*, 5 minutes, RT).
4. The supernatant was aspirated and the pellet was resuspended in 0.5 mL of Stain Buffer. The tube was vortexed and stored at 2–8°C in the dark until acquired.

Instrument Setup

The daily setup of a BD FACSVerse system is a single step: Performance QC using CS&T research beads. For the assay described in this application note, default LW settings were used, which provided optimal positioning of the cells on scale for FSC vs SSC scatter and all fluorescence parameters. The default measured SOVs were used for compensation. Of note, in addition to the default settings, user-defined tube settings and assays can also be created and can be used repeatedly on the same instrument or transported to other BD FACSVerse systems. The normal daily workflow using default LW or LNW settings is presented in Figure 2.

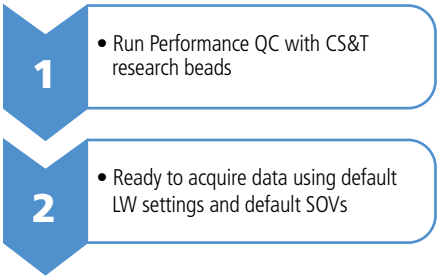

Figure 2. Workflow for daily instrument setup using default settings.

One of the markers in the panel, CD4, was conjugated to the tandem dye PE-Cy7, the SOVs for which can vary from lot to lot. To measure lot-specific SOVs for CD4 PE-Cy7, it was initially added to the existing SOV matrix. Only the PE-Cy7 controls were acquired, not BD CompBead controls for the rest of standard fluorochromes in the panel.

Setting up an assay on a BD FACSVerse system on day 1 and instrument 1
An assay was created on day 1 on instrument 1 using the LW tube settings, along with a worksheet containing plots, gates, and stopping criteria. The workflow shown in Figure 3 was used to initially create the assay on day 1 and on instrument 1.

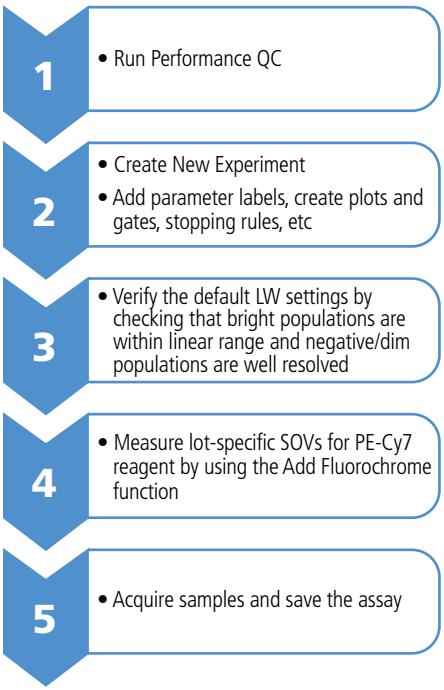

Figure 3. Workflow for assay setup on day 1 and on instrument 1.

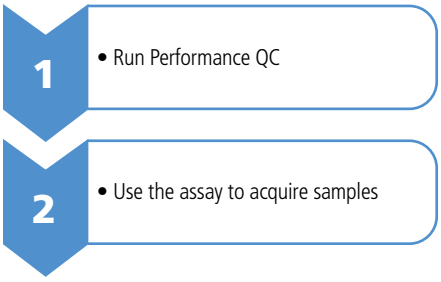

Figure 4. Workflow for running the same assay on different days.

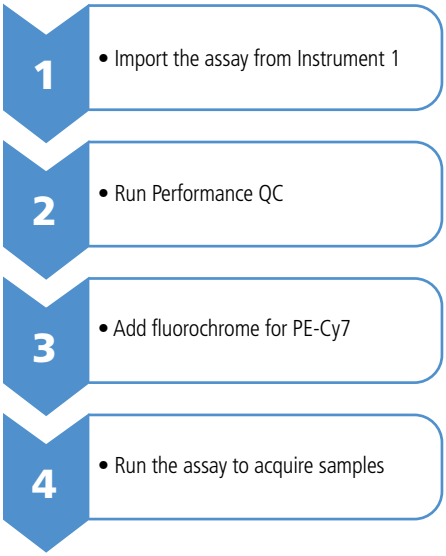

Figure 5. Workflow for running the same assay on different instruments.

Running the same assay on different days

After the initial setup described in the preceding section, the assay was used on different days. The workflow presented in Figure 4 was used to run the same assay on the same instrument (1) on day 7 and day 25.

Running the same assay on different instruments with different operators

The assay from instrument 1 was exported and then imported into BD FACSuite software on instruments 2 and 3. The workflow shown in Figure 5 was used to acquire data using the imported assay on instruments 2 and 3.

Results

Figure 6 shows an overview of the T-memory effector assay and the gating strategy that was used to identify specific populations. To demonstrate the standardization of assays and consistency of data on different days on the same instrument, blood samples from two different donors were acquired on days 1, 7, and 25 on a single instrument (instrument 1). Further, to demonstrate the portability of the assay, the T-memory effector assay was created on one instrument and then exported to two other instruments and run by different operators.

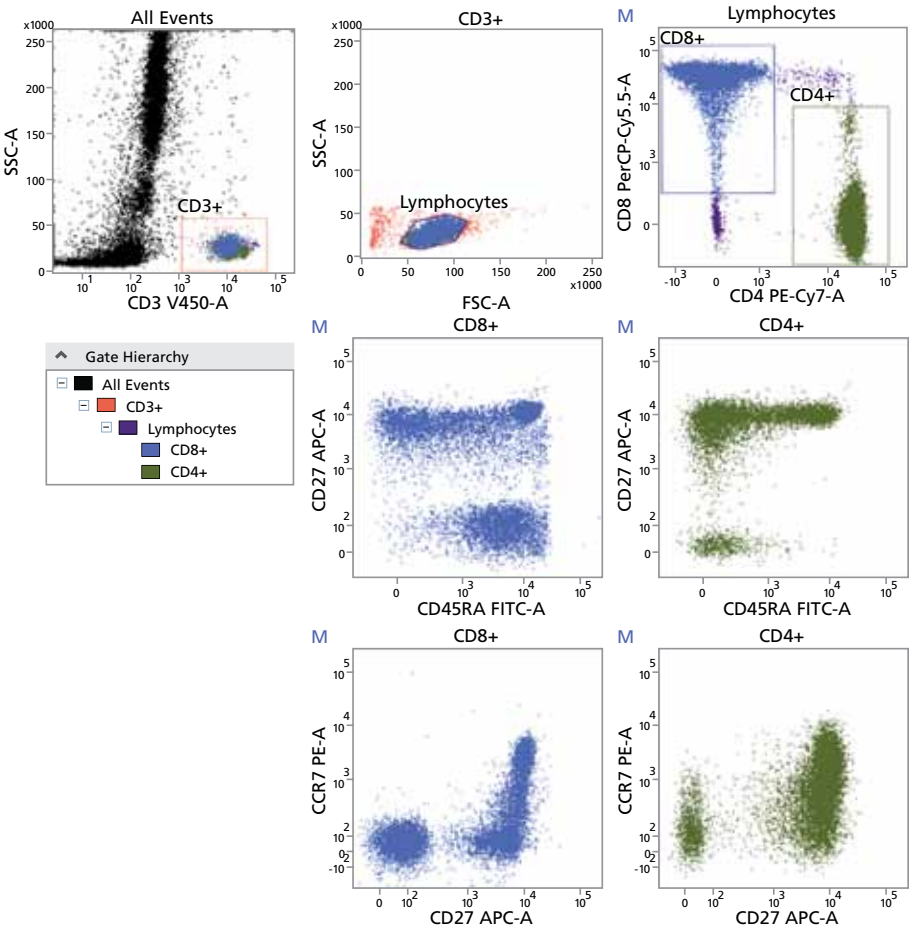

Figure 6. T-memory effector assay gating strategy.

Comparison of MFIs on Different Days and on Different Instruments

For comparison of data from two donors on different days and across different instruments, a representative marker with a fluorchrome excited by each laser was selected. The MFI data from CD4 PE-Cy7, CD27 APC, and CD3 V450 from positive populations on different days and from different instruments is presented in Table 1 along with %CV values. The percent coefficient of variation (%CV) values of MFI data were less than 10% (range 2.16–5.45%) when data was acquired on different instruments. When the data from same donor was acquired on different days, the %CV ranged from 1.77–19.77. The data from CD4 PE-Cy7, CD27 APC, and CD3 V450 in the form of stacked histograms overlays is presented in Figure 7, which also clearly shows the reproducibility of data from all the populations with minimal day-to-day and instrument-to-instrument variation.

Table 1. MFI comparison on different days and on different instruments.

Donor	Marker	Days (on instrument 1)				Instruments (on day 1)			
		1	7	25	%CV (across days)	1	2	3	%CV (across instruments)
1610	CD4 PE-Cy7	32,806	32,690	34,599	**3.21**	32,806	32,479	30,818	**3.33**
	CD27 APC	7,952	7,921	7,697	**1.77**	7,952	8,412	7,544	**5.45**
	CD3 V450	11,736	13,963	13,313	**8.81**	11,736	11,649	11,267	**2.16**
5185	CD4 PE-Cy7	30,372	31,350	33,379	**4.84**	30,372	30,372	28,566	**3.5**
	CD27 APC	8,697	8,028	8,575	**4.22**	8,387	8,697	8,149	**3.27**
	CD3 V450	14,722	10,157	11,263	**19.77**	14,539	14,722	14,039	**2.45**

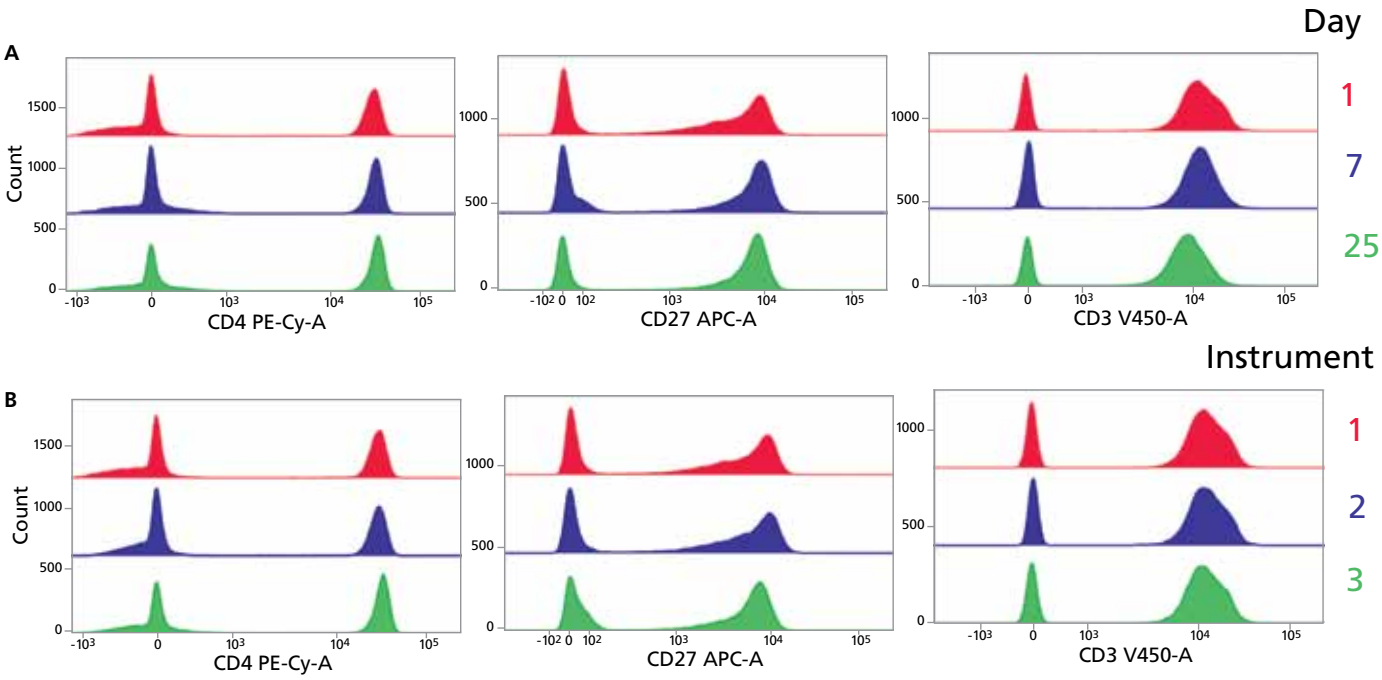

Figure 7. Stacked histogram overlays for CD4 PE-Cy7-A, CD27 APC-A, and CD3 V450-A from donor 5185.

A. Acquired on instrument 1 on different days. B. Acquired on different instruments by different operators on day 1. The data is from lymphocytes gated on FSC-A and SSC-A.

Comparison of Compensation on Different Days and on Different Instruments

To demonstrate the consistency of automated compensation on different days and across different instruments, we compared the automated compensation applied by BD FACSuite software on different days. Figure 8 shows the comparison of data on different days and on different instruments in the form of contour plots of fluorochrome combinations known to have SOVs between them: FITC into PE, PE-Cy7 into PE, and PerCP-Cy5.5 into PE. The data in the plots clearly shows the consistency of compensation on different days and on different instruments.

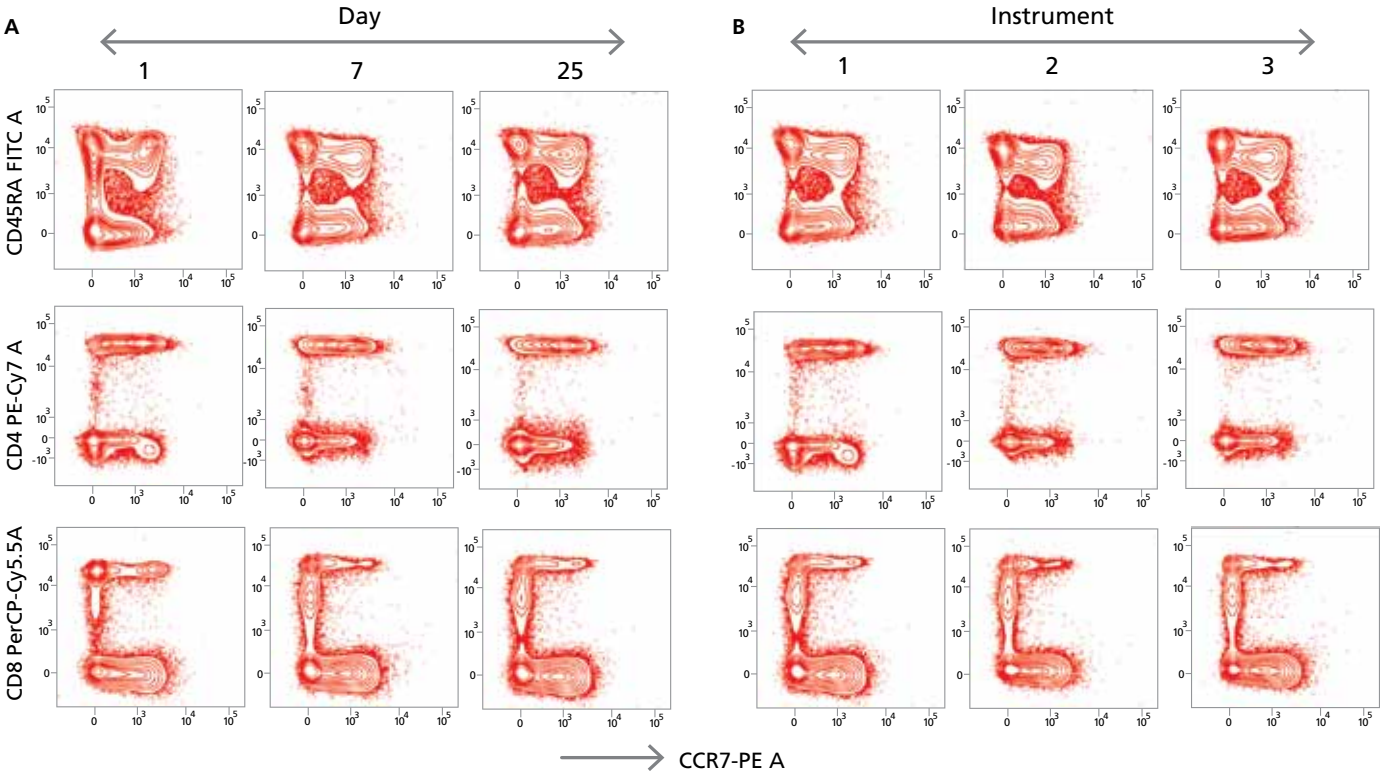

Figure 8. Contour plots of CD45RA FITC-A vs CCR7 PE-A, CD4 PE-Cy7 vs CCR7 PE-A, and CD8 PerCP-Cy5.5-A vs CCR7 PE-A to demonstrate the consistency of compensation.

Panel A. *Data from donor 5185 acquired on instrument 1 on different days.* ***Panel B.*** *Data from donor 5185 acquired on different instruments by different operators on day 1.*

Precision of MFI Targets and Use of Consistent Gates on Different Days and on Different Instruments

The reproducibility of the instrument setup and assays was further confirmed by identifying and gating specific populations P1 and P2 in CD27 APC-A vs CCR7 PE-A plots. The gates P1 (double-negative population) and P2 (bright double positive population) are shown as indicators and were not moved after initial assay setup. When the same sample from the healthy donor was run on different days, the populations of interest were within the gates that were initially set up. Because of the consistency of MFI target values ensured by the BD FACSVerse system setup, the data truly represented the biological variations over the period of time without any variation due to the instrument. Also, when the assay was transported to two other instruments and the same donor sample was run on all three instruments on the same day, the double-negative and bright double positive populations again were in the respective gates without any variation. This clearly shows the robustness of instrument setup, reproducibility of assays, and overall senstivity of the BD FACSVerse system to place the specific populations in pre-defined gates without any need for adjustment of PMTVs and the gates on the worksheets.

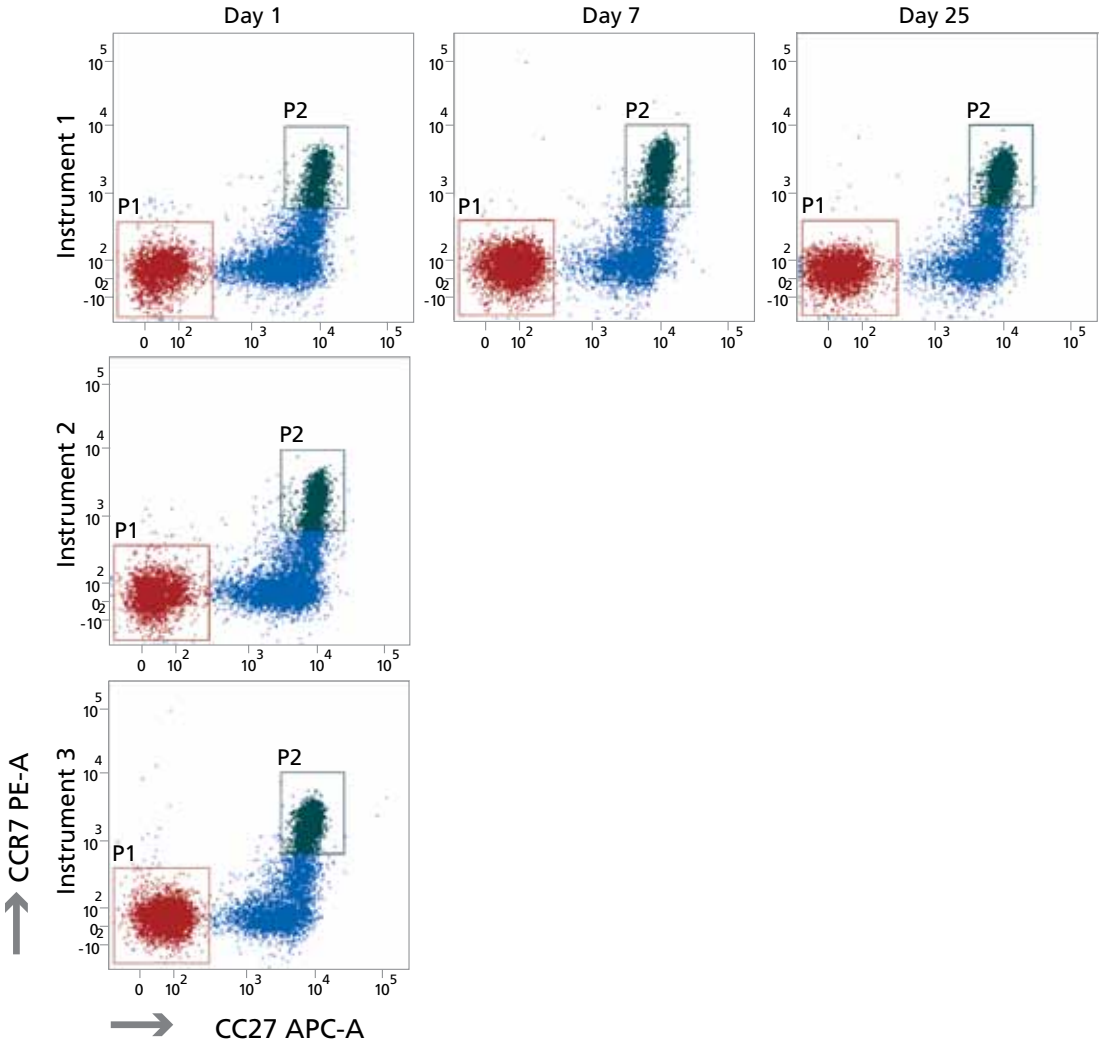

Figure 9. CD27 APC-A vs CCR7-PE-A plots from the CD8⁺ population showing the precision of MFI targets and consistency of gating on instrument 1 on different days and on on different instruments with different operators on day 1.

Discussion

This application note presents a proof of principle example on the BD FACSVerse system to address the major questions about instrument setup, standardization, and portability of assays that flow cytometry users commonly ask.

The basic instrument setup of a flow cytometer involves adjustment of the PMTVs and compensation. The correct adjustment of PMTVs for each fluorescence detector is necessary, since these adjustments can affect the sensitivity and separation of dim versus bright fluorescent signals. When the PMTVs are set too low, dim populations might not be well resolved from unstained cells. Conversely, when the PMTVs are set too high, very bright fluorescent markers might exceed the linear range of a detector, which might then lead to inaccurate compensation. Therefore, the first step in any flow cytometry assay is the setup and QC to check performance of the instrument every day. There are a variety of fluorescent particles that are commonly used to check instrument performance, but most of these methods are manual, laborious, and time consuming. The BD FACSVerse system requires only a single-step Performance QC with CS&T research beads once a day to measure and track the instrument performance. The Performance QC ensures that PMTVs are automatically set up to achieve the MFI target values of the assays. This daily automatic adjustment enables the reproducibility of an assay when run on different days. By using an example of a T-memory effector assay with default LW settings, we have demonstrated that when samples from a single donor were run on different days, similar MFIs of various markers in the panel were achieved. Furthermore, the populations of cells were on the scale in all the acquisition plots, and there was no need to adjust the gates on the plots on different days.

Compensation is an essential component of multicolor flow cytometry, and to obtain accurate results from each fluorochrome, the SOVs must be calculated. This is normally performed by running compensation controls along with the test samples every time an assay is run. The BD FACSVerse system provides a very easy and automated method for compensation. The SOVs for all standard fluorochromes are measured and stored in the system and can be used for 30 days. These SOVs are automatically applied to each tube in an assay without running compensation controls on a daily basis. For assays with tandem dyes, SOVs can vary from lot to lot because these fluorochromes are subject to degradation with prolonged exposure to light, elevated temperatures, and fixatives. When using these fluorochromes, lot-specific compensation is recommended. In the panel that was used in this application note, PE-Cy7 was the tandem fluorochrome and needed lot-specific SOVs. The SOVs for PE-Cy7 were added to the existing SOV matrix of standard fluorochromes simply by running a PE-Cy7 control—without the need to run the other fluorescence controls again. The measured SOVs for standard fluorochromes along with lot-specific SOVs for PE-Cy7 in the panel produced consistent compensation when samples from the same donor were run on day 1, 7, and 25.

Assay portability is one of the fundamental needs for multisite and collaborative projects for which samples need to be run in different laboratories and by different operators with different levels of expertise, and for which the data needs to be compared. BD FACSuite software in the BD FACSVerse system provides the ability to export assays to other BD FACSVerse systems, thereby providing transportability of assays across different laboratories. When an assay is exported from one instrument and imported into another, the Performance QC step automatically adjusts the imported assays to match the target MFI values of the assays on the new instrument. To show the proof of principle of portability, in this study we exported the T-memory effector assay from one instrument and imported it into BD FACSuite software on two other instruments. Minimal

variations were observed in MFI data for various markers. There was no need to adjust PMTVs when the assay was transported to other instruments, which is evident from the data resolution. In this case, the populations consistently fell into the gates even when the assay was run on different instruments. The automated measured SOVs that reside in the each instrument were consistent when the data from the same donor was compared across the three instruments.

Conclusions

Ease of use of daily instrument setup, reproducibility of data and the portability of assays are the essential needs of flow cytometry researchers. Using an example of a T-memory effector assay, we have demonstrated the ease of use, consistency of performance, automated compensation, reproducibility, and portability features of the BD FACSVerse system. The system's capabilities can be applied to other flow cytometry assays. These features are very useful for generating reproducible data for longitudinal and multisite studies in which the same assay is used to acquire data on different days, on different instruments, and by different operators with different levels of expertise.

References

1. Maecker HT, McCoy JP, Nussenblatt R. Standardizing immunophenotyping for the human Immunology Project. *Nat Rev Immunol.* 2012;12:191-200.

Tips and Tricks

- LW and LNW default settings are adequate for most blood-based assays using standard fluorochromes.
- The measured SOVs are automatically applied when standard fluorochromes are used and can be reliably reused for 30 days.
- For tandem dyes, we recommend using the Add Fluorochrome option in BD FACSuite software for calculation of lot-specific SOVs.
- For user-defined assays, we recommend performing Assay & Tube Settings Setup before the data acquisition to ensure application of the correct target MFIs.

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