Setup Procedure for BD FACSAria™, FACSCanto™ and LSR Series

This part of the guide applies to BD digital flow cytometers using FACSDiva™ software version 6.0 and above.

For the BD FACS machines running FACSDiva™, use the PE channel for reporter and the APC channel for beads classification. In general, there is no need for compensation between these channels if the machine is set up properly following the setup procedure described below.

This setup procedure is required under the following situations:

- You are running the LEGENDplex kit for the first time.
- It has been over a month since the procedure was last performed.
- Your flow cytometer has been serviced since you last performed this procedure.

This setup process is not needed if you have run this experiment before and have access to a saved experiment template (The settings will be saved in the final step of this setup procedure and any settings saved can be imported to a new experiment. Please refer to Step 2 and Step 3.9 below).

1. Start up the Instrument

Perform instrument startup and verification check following the manufacturer's recommendations.

2. Obtain a Template for Data Acquisition

A template for FACSDiva™ is a worksheet with density plots that allows the user to perform machine setup and data acquisition.

If a template is not yet available, create a new template by following the instructions in step 2. After a template is created, save the file in D:\BDExport\Templates\Experiment. Do not change the name of the Templates folder.

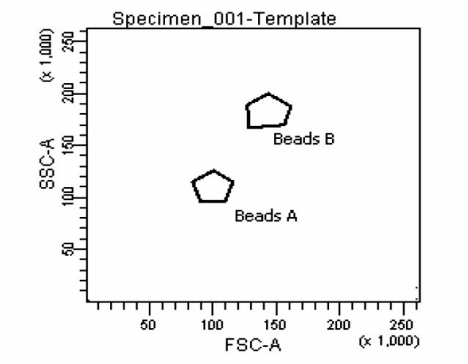
If you have already created a template for the flow cytometer, open that template and proceed to Step 3. To open an existing template, select Experiment> New Experiment. A list of templates saved in D:\BDExport\Templates\ Experiment will pop up. Select the desired template from the list.

To create a new template, follow the instructions below:

2.1 From the BD FACSDiva™ software, go to Experiment > New Experiment.

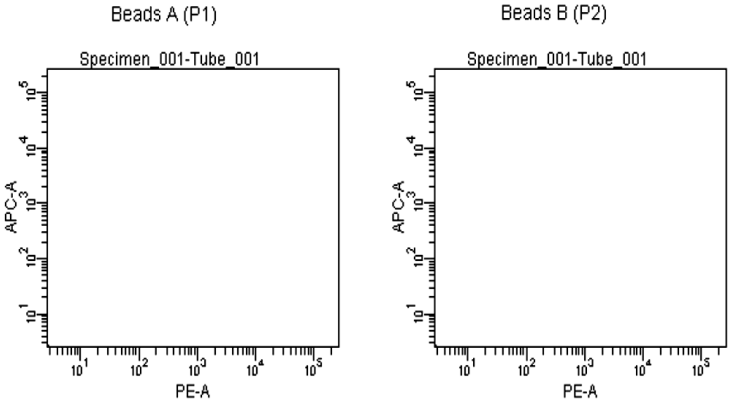
2.2 In the global worksheet, open the worksheet. Create a dot plot with FSC (forward scatter) for X-axis and SSC (side scatter) for Y-axis. **Be sure to set FSC and SSC to linear mode.** Create two gates and label them Beads A and Beads B (Figure 19).

Figure 19.



2.3 Create two dot plots with PE for X-axis, APC for Y-axis (Figure 20), gated on Beads A (left panel below) and Beads B (right panel below), respectively. Create one dot plot with FITC for X-axis, APC for Y-axis, gated on Beads A and Beads B (graph not shown). **The plots should all be in log mode**.

Figure 20.



2.4 Save the document as “LEGENDplex Template for FACS Diva” in D:\BDExport\Templates\Experiment and proceed to the next step of setup.

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3. Set up PMT Voltages

The Setup Beads 3: Raw Beads are used to set up the PMT voltage of the classification channel APC, reporter channel PE, and FITC channel. The Setup Beads 1: FITC Beads and 2: PE Beads are not needed for this setup because no compensation is required if the setup procedure described here is closely followed.

Follow the instructions below for setting up the PMT settings:

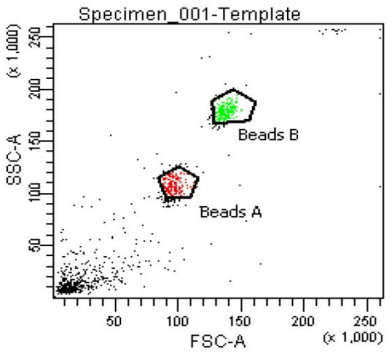
3.1 Vortex the vial of Raw Beads for 30 seconds to resuspend the beads.

3.2 Transfer 400 µL of the Raw Beads to a fresh FACS tube.

3.3 Set the flow cytometer flow rate to low. Run the Raw Beads. Adjust the settings for FSC and SSC so that both bead populations are visible (Figure 21).

Pause and restart acquisition frequently during the setup procedure to refresh the beads populations after adjusting settings.

Figure 21.

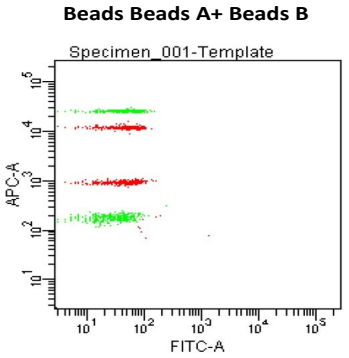


3.4 Continue adjusting the settings so that Beads A and Beads B are well separated and the FSC and SSC readings are >50 (x1000).

3.5 Move the gates for Beads A and Beads B so that the smaller beads fall into Beads A gate and the larger beads fall into Beads B gate (Figure 21).

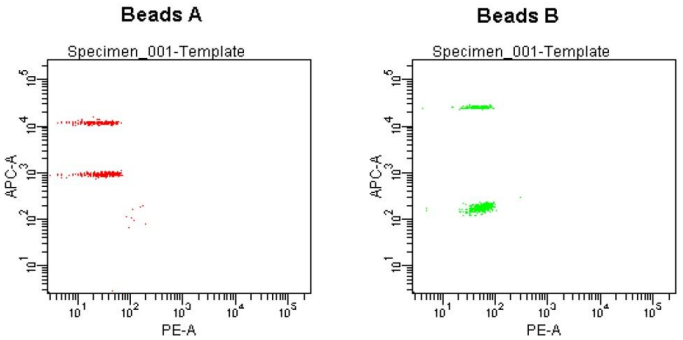
3.6 Adjust the FITC setting so that the FITC signal for the majority of beads is between $1x10^1$ and $1x10^2$ (Figure 22).

Figure 22.



3.7 Adjust the PE setting so that the PE signal for the majority of beads is between $1x10^1$ and $1x10^2$ (Figure 23).

Figure 23.



3.8 Adjust the APC settings so that the the APC fluorescence intensities of all bead populations are between $1x10^2$ and 5×10^4 (Figure 23).

3.9 Save the document again for future use.

To save your assay-specific settings, in the browser, right-click Cytometer Settings and select Save to **Catalog**. Name the file, and then click OK. To import the saved setting for a new experiment, right click on cytometer settings and select import settings.

3.10 The flow cytometer is now ready for sample analysis.