

Setup Procedure for FACSCalibur™ with Dual Lasers

For a dual laser FACSCalibur™, use FL2 for reporter and FL4 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.

1. Start up the Instrument

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

2. Obtain a Template for Data Acquisition

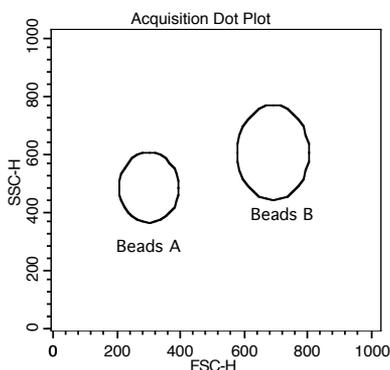
A template for FACSCalibur™ is a document with density plot that allows the user to perform machine setup and data acquisition.

If you have already created a template for the flow cytometer, open that template and proceed to Step 3.

If a template is not yet available, create a new template by following the instructions below:

- 2.1 From the BD CellQuest data acquisition software, go to File → new document.
- 2.2 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 4).

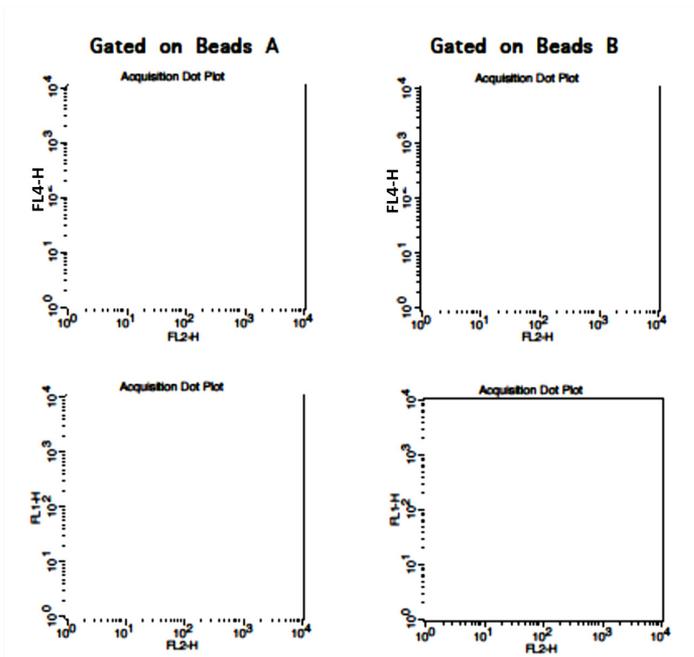
Figure 4.



- 2.3 Create 4 fluorescent dot plots as shown below (Figure 5) with FL2 for X-axis, FL4 or FL1 for Y-axis. For the fluorescent dot plots, gate on Beads A (dot plots on the left panel below) and Beads B (dot plots on the right panel below). **The dot plots should be in log mode.**

- 2.4 Save the new document as “LEGENDplex Template for FACSCalibur Dual Laser” and proceed to the next step of the setup.

Figure 5.



3. Set up the PMT Voltages

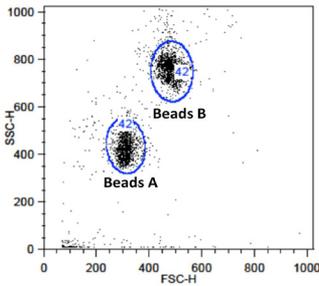
The Setup Beads 3: Raw Beads are used to set up the PMT voltage of the classification channel (FL4 /APC) and channel FL1. The Setup Beads 2: PE Beads are used to set up the PMT voltage of the reporter channel (FL2/PE). The Setup Beads 1: FITC Beads are not needed for this setup.

Follow the instructions below for setting up the PMT settings:

- 3.1 Vortex the vial of the Raw Beads for 30 seconds to resuspend the beads.
- 3.2 Transfer 400 μ L of the Raw Beads to a fresh FACS tube.
- 3.3 Set the flow cytometer flow rate to low. In setup mode, run the Raw Beads. Adjust the settings for FSC and SSC so that both bead populations are visible (Figure 6).

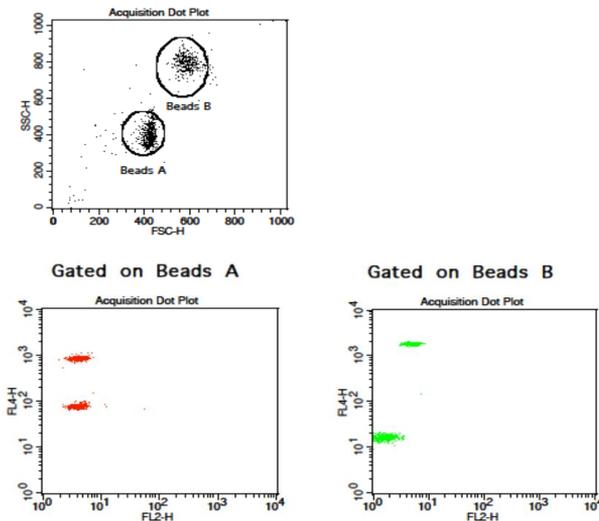
Pause and restart acquisition frequently during the setup procedure to refresh the bead populations after adjusting settings.

Figure 6.



- 3.4 Continue adjusting the settings so that Beads A and Beads B are well separated and the FSC and SSC readings are >200.
- 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 6).
- 3.6 Adjust the FL1 setting so that the FL1 signals for all beads are between 1×10^0 and 1×10^1 (Note: This step is not required, but is recommended).
- 3.7 Adjust the FL4 setting so that the FL4 signals for all beads are between 1×10^1 and 5×10^3 (Figure 7).

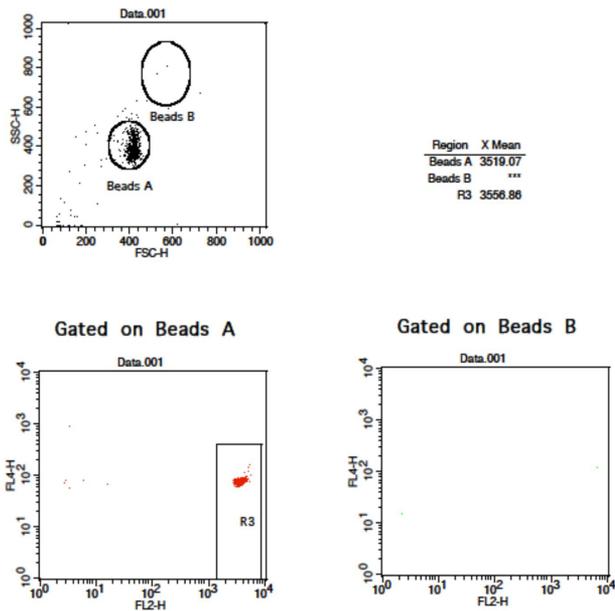
Figure 7.



- 3.8 Vortex the vial of Setup Beads 2: PE Beads for 30 seconds to resuspend the beads.

- 3.9 Transfer 400 μ L of the PE Beads to a fresh FACS tube.
- 3.10 Replace the Raw Beads tube from the flow cytometer with the PE beads tube.
- 3.11 Set the flow cytometer flow rate to low. In setup mode, run the PE Beads. Note: PE beads are only of small size, falling in the Beads A gate (Figure 8).
- 3.12 Adjust the FL2 setting so that the median fluorescence intensity of the PE beads falls between the lot-specific range labeled on the PE Beads vial (Figure 8, gate R3).

Figure 8.



- 3.13 Save the document again for future use.
- 3.14 The flow cytometer is now ready for sample analysis.