

Setup Procedure for FACSCalibur™ with a Single Laser

For a single laser FACSCalibur™, use FL2 for reporter and FL3 for beads classification. Compensation is needed to properly set up the instrument.

1. Start up the Instrument

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

2. Obtain a Template for Data Acquisition

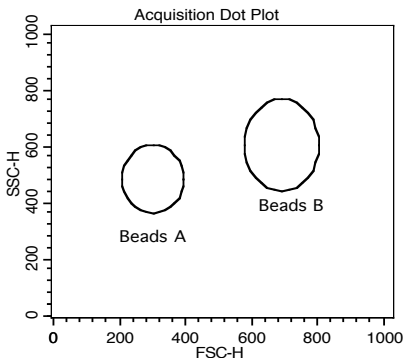
A template for FACSCalibur™ is a document with density plot that allows the user to perform machine setup and data acquisition.

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:

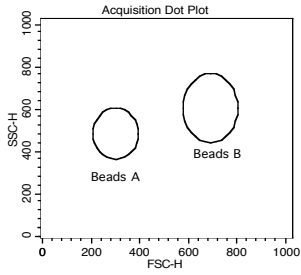
- 2.1 From the BD CellQuest data acquisition software, go to File → new document.
- 2.2 Create a dot plot with FSC (forward scatter) for X-axis and SSC (side scatter) for Y-axis. **Be sure to set FSC and SSC to linear mode.** Create two gates and label them Beads A and Beads B (Figure 9).

Figure 9.

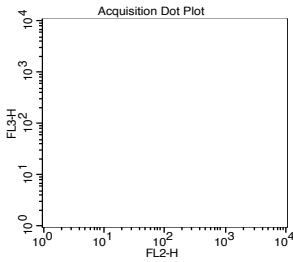


- 2.3 Create 4 fluorescent dot plots as shown below (Figure 10) with FL2 for X-axis, and FL3 or FL1 for Y-axis. For the fluorescent dot plots, gate on Beads A (dot plots on the left panel below) and Beads B (dot plots on the right panel below). **The dot plots should be in log mode.**

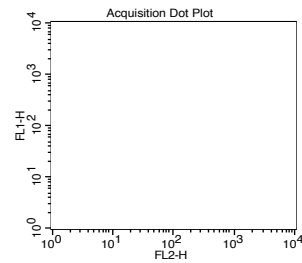
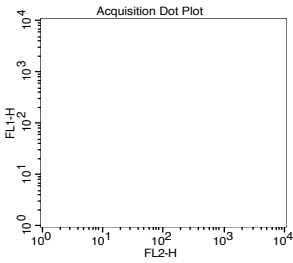
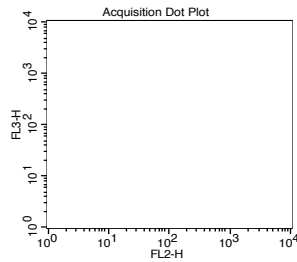
Figure 10.



Gated on Beads A



Gated on Beads B



2.4 Set all compensations to zero.

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

3. Set up PMT Voltages and Compensation

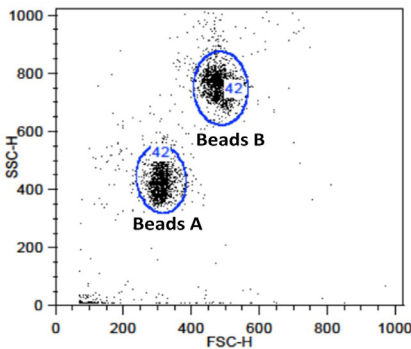
The Setup Beads 3: Raw Beads are used to set up the PMT voltage of the classification channel FL3 and channel FL1. The Setup Beads 2: PE Beads are used to set up the PMT voltage of the reporter channel FL2. All three setup beads are needed for setting up compensation.

Follow the instructions below for setting up the PMT settings and compensation:

- 3.1 Vortex the vial of the 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. In setup mode, run the Raw Beads. Adjust the settings for FSC and SSC so that both beads populations are visible (Figure 11).

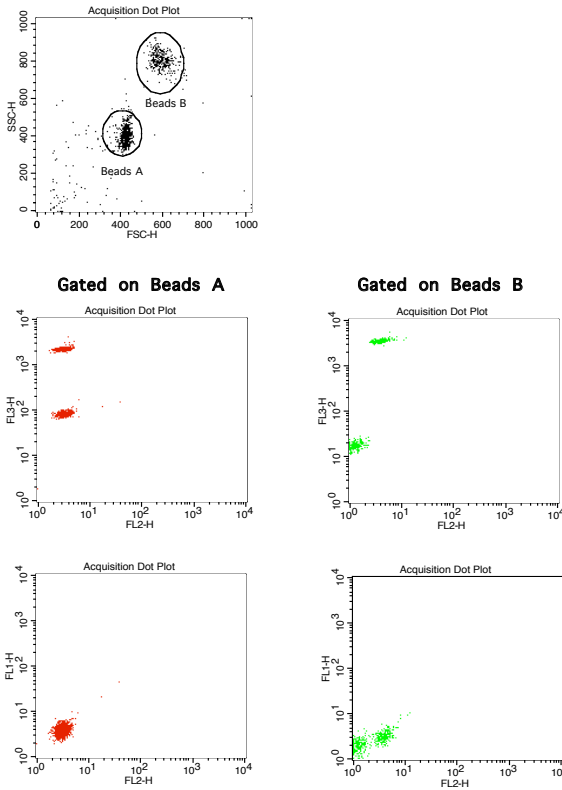
Pause and restart acquisition frequently during the setup procedure to refresh the bead populations, after adjusting settings.

Figure 11.



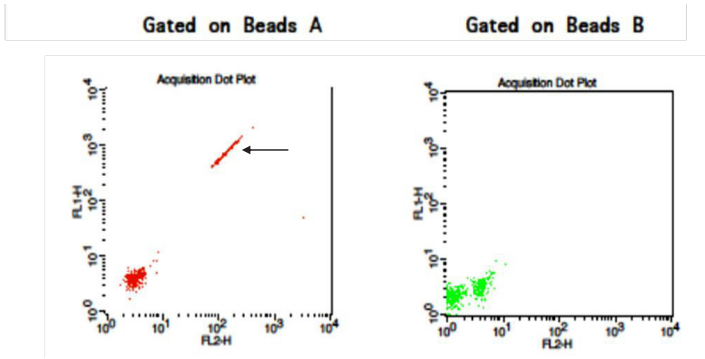
- 3.4 Continue adjusting the settings so that Beads A and Beads B are well separated and the FSC and SSC readings are >200 .
- 3.5 Move the gates for Beads A and Beads B so that the smaller beads fall into Beads A gate and the larger beads fall into Beads B gate (Figure 11).
- 3.6 Adjust the FL1 setting so that the FL1 signals for all beads are between 1×10^0 and 1×10^1 (Figure 12).

Figure 12.



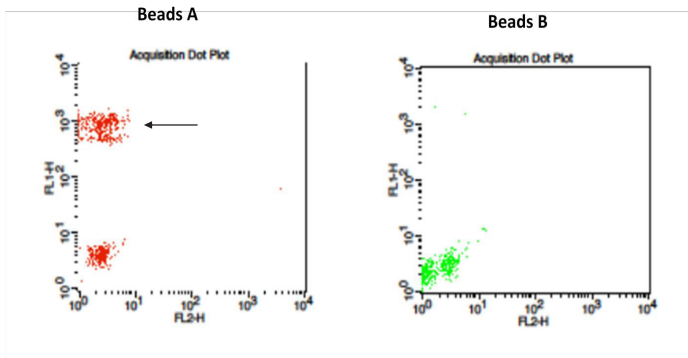
- 3.7 Adjust the FL2 settings so that the FL2 signals for all beads are between 1×10^0 and 1×10^1 (Figure 12).
- 3.8 Adjust the FL3 setting so that the FL3 signals for all beads are between 1×10^1 and 5×10^3 (Figure 12).
- 3.9 Vortex the vial of the Setup Beads 1: FITC Beads for 30 seconds to resuspend the beads.
- 3.10 Transfer 200 μL of the FITC Beads to a fresh FACS tube. Add 200 μL of Raw Beads and mix well (this will generate FITC-positive and FITC-negative populations of beads and is needed for proper compensation).
- 3.11 In setup mode, run the mixed FITC and Raw Beads.
- 3.12 On the FL1 vs FL2 dot plot (Figure 13), the beads will display as FITC-negative and FITC-positive populations (indicated by an arrow).
Note: FITC beads are only of small size, falling in the Beads A gate.

Figure 13.



3.13 Adjust the FL2-%FL1 compensation setting (e.g., FL2-FL1 = 20%) so that the FITC-negative and FITC-positive populations have similar mean FL2 fluorescence intensities (Figure 14).

Figure 14.



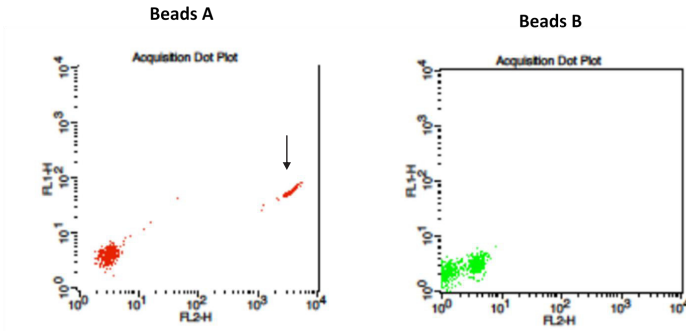
3.14 Vortex the vial of PE Beads for 30 seconds to resuspend the beads.

3.15 Transfer 200 μ L of the PE Beads to a fresh FACS tube. Add 200 μ L of Raw Beads and mix well (This will generate PE-positive and PE-negative populations of beads and is needed for proper compensation).

3.16 In setup mode, run the mixed PE and Raw Beads.

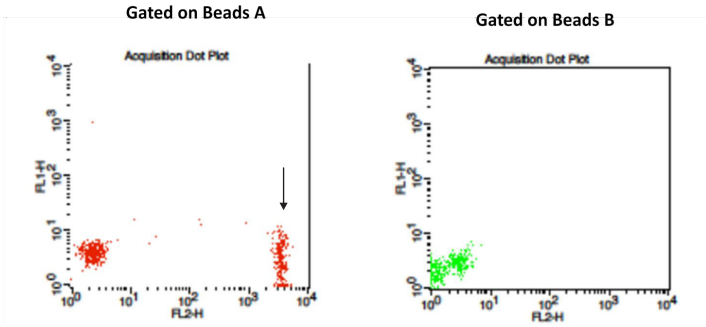
3.17 On the FL1 vs FL2 dot plot (Figure 15), the beads will display as PE-negative and PE-positive populations (indicated by an arrow). Note: PE beads are only of small size, falling in the Beads A gate.

Figure 15.



- 3.18 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 16).
- 3.19 Adjust the FL1-%FL2 compensation setting (e.g., FL1-FL2 = 1.5%) so that the PE-negative and PE-positive populations have similar mean FL1 fluorescence intensities (Figure 16).

Figure 16.



- 3.20 On the FL3 vs FL2 dot plot (Figure 17), the beads will display as PE-negative and PE-positive populations (indicated by an arrow).
- 3.21 Adjust the FL3-%FL2 compensation setting (e.g., FL3-%FL2= 40%) so that the FL3-low population and the PE-positive population have similar median FL3 signal (Figure 18).

Figure 17.

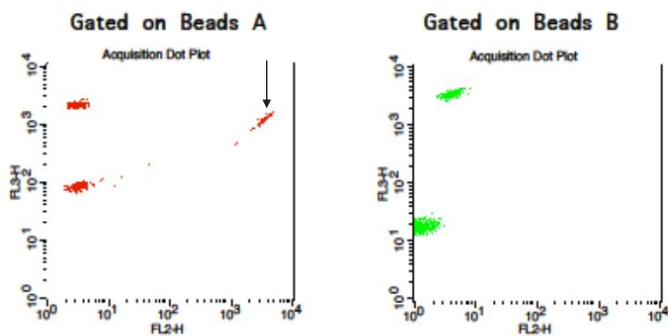
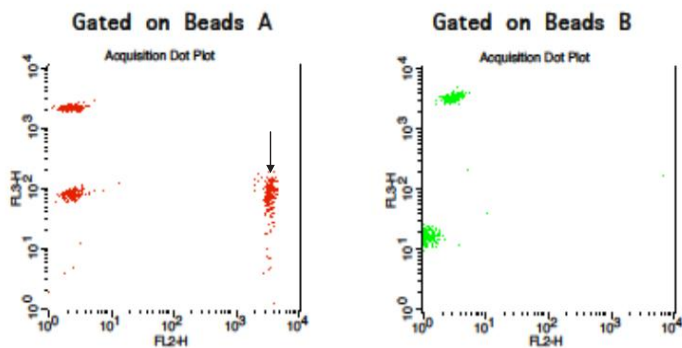


Figure 18.



3.22 Save the document again for future use.

3.23 The flow cytometer is now compensated and ready for sample analysis.