Setup Procedure for Beckman Coulter CytoFLEX Flow Cytometer

This part of the guide applies to Beckman Coulter flow cytometers using Cyto-Expert software version 1.2.8 and later.

For CytoFLEX flow cytometers, the PE channel is used for reporter and the APC (or AF647) channel is used 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.

- Your flow cytometer has been serviced since you last performed this procedure.

This setup procedure is not needed if you have run this experiment before and have access to a saved template (The settings can be saved in the final step of this setup procedure and any settings saved can be imported to a new experiment. Please refer to section 3 below).

1. **Start up the Instrument**

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

2. **Creating a New Template for Data Acquisition**

   A template is a file (with ".xitm" extension) generated in the CytoExpert software with density plots, pre-defined gates and settings that allow the user to skip the setup procedure and perform sample acquisition directly.

   If a template is not available, create a new template following the instructions below. **If a template has been created, please skip this setup and proceed directly to Section 3.**
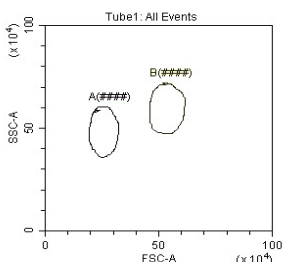
   2.1 Set up Density Plots and Gates

   2.1.1 From the CytoExpert software home screen, click Cytometer >> Detector configuration. From the popup window, select default or any desired detector configuration >> Set as current >>OK.

   2.1.2 From the CytoExpert software home screen, click New Experiment or go to File and select "New experiment".

   2.1.3 A popup window promotes creating a new folder for data

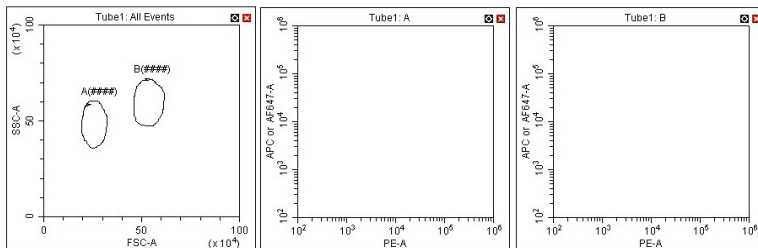storage. Save the folder in a desired location. Change the folder name if desired.

2.1.4 From the toolbar, click the density plot sign and a dot plot will appear with FSC-A (forward scatter) for X-axis and SSC-A (side scatter) for Y-axis. **Set the FSC-A and SSC-A to linear mode.** To change the mode, click on the density plot, then right click the mouse and select property.

2.1.5 Create two gates and label them as gate A and gate B. Gate A is for A Beads (smaller beads) and gate B is for B Beads (larger beads, Figure24).

Figure 24

2.1.6 Create two dot plots with PE for X-axis, APC (or AF647) for Y-axis (Figure 25), gated on A Beads (middle panel below) and B Beads (right panel below), respectively. To gate a specific population for a dot plot, click on that dot plot, right click the mouse on the name of the dot plot and select the desired gate from the gate list. **The plots should all be in log mode**. To change the mode, click on the density plot, right click the mouse and select property. Also under property, **always set the maximal PE-A and APC-A (or AF647) value to 1,000,000 and the minimum value to 100 for consistent setup.**

Figure 25

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2.1.7 Save the document as "LEGENDplex Template for CytoFLEX in a desired folder and proceed to the next step of the setup.
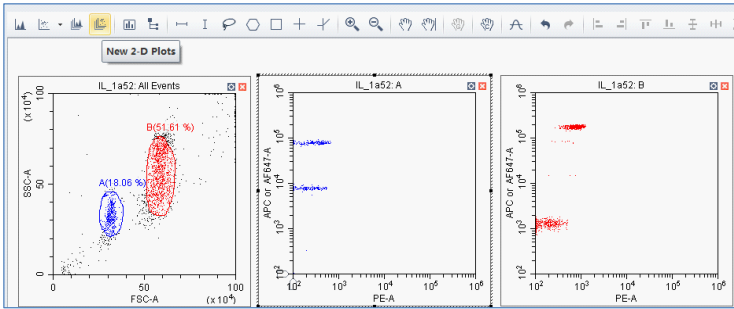
2.2 Set up PMT Voltages

The Setup Beads 3: Raw Beads are used to set up the PMT voltages of the classification channel APC (or AF647) and reporter channel PE. The Setup Beads 1: FITC Beads is not needed for this setup because no compensation is required if the setup procedure is closely followed. The Setup Beads 2: PE beads is not required, but can be used as a reference for optimal PMT voltages for PE channel.

Follow the instructions below for setting up the PMT voltages:

2.2.1 Click on the Cytometer tab, open Acq. Setting.

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

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

2.2.3 Set the flow cytometer flow rate to low. Run the Raw Beads. Adjust the gains in the Acq. Settings for FSC-A and SSC-A so that both bead populations are visible (Figure 26, left panel). **Alternatively, from the toolbar, click the hand sign with letter "G" (gain) and use the mouse to drag the populations to the middle of the dot plot.** Pause and restart acquisition frequently during the setup procedure to refresh the bead populations after adjusting settings.

2.2.4 Continue adjusting the settings so that A Beads and B Beads are well separated and the FSC and SSC readings are $\geq 20 \times 10^4$. This is to ensure that the bead populations are well separated from debris.

2.2.5 Move the gates for A Beads and B Beads so that the smaller beads fall into A gate and the larger beads fall into B gate (Figure 26, upper left panel)

2.2.6 Adjust the APC settings so that the the APC fluorescence intensities of all bead populations are between 1×10^3 and 3×10^5 (Figure 26, upper middle and right panels).

2.2.7 Adjust the PE setting so that the PE signal for majority of the beads is between 1×10^2 and 1×10^3 (Figure 26, upper middle and right panels).
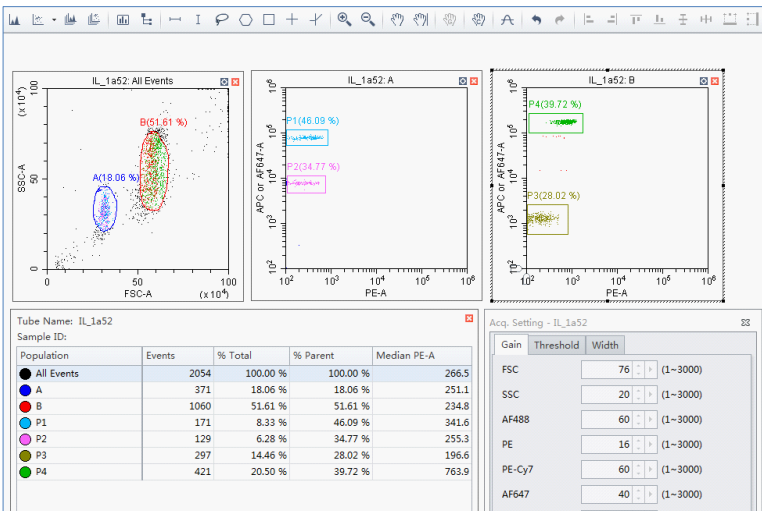
Figure 26

2.2.8   Gate on the 4 populations of the setup beads 3 (Figure 27, gate P1, P2, P3 and P4) and generate statistics for the 4 populations by clicking on the the "bar graph"sign. Continue Adjust the PE channel Gain so that the median flourescence intensity for the 4 populations are between ~200-800 (Figure 27, Statistic table). In this example, P1=341.6, P2=255.3, P3=196.6 and P4=763.9.
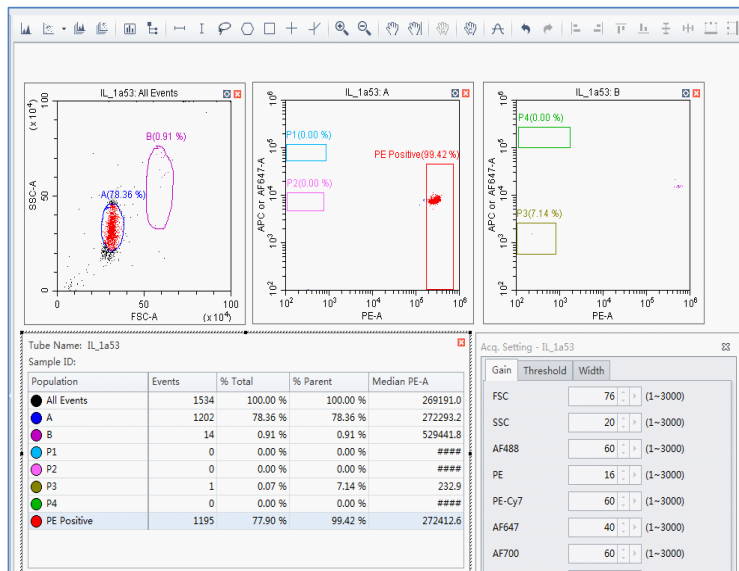
Note: Setting an optimal PMT voltage (Gain) of the PE channel is critical to obtain the best standard curve. If the voltage is set too low, then the low end of the standard curve may plateau, resulting in low sensitivity of the assay. If the voltage is set too high, the high end of the standard curve may plateau.

Figure 27

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2.2.9 Vortex the vial of Setup Beads 2: PE Beads for 30 seconds to resuspend the beads. Transfer 400 μL of the PE Beads to a fresh FACS tube.

2.2.10 Replace the Raw Beads tube from the flow cytometer with the PE beads tube.

2.2.11 Set the flow cytometer flow rate to low and run the PE Beads. Note: PE beads are only of small size, falling in the A gate (Figure 28, upper left panel).

2.2.12 Check the median fluorescence intensity of the PE beads. The Median PE siganl of the beads shoud be between 2.5×10^5-3.0×10^5 (Figure 27, upper middle panel PE positive gate).

Note: The median fluorescence intensity range for PE beads specified above is for reference only and may change from lot to lot. Setup Beads 2: PE beads is not required to set the PMT voltage (Gain) of the PE channel.

2.2.13 Save the template and proceed to the next step of the setup.
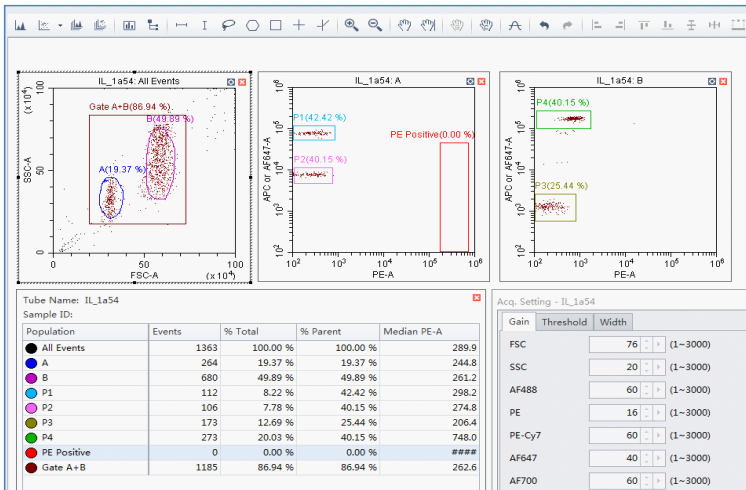
Figure 28

2.3 Gate out Debris and Set Number of Events to Record

Sometimes samples may contain large amounts of debris that can jeopardise both the sample acquisition and data analysis. It is therefore important to gate out the debris and set the correct number of events to be recorded following the instructions below.

2.3.1   Draw a large gate including both A and B beads. Name it gate A+B as shown below (Figure 28, upper left panel)

2.3.2   Set events to record as 300 events per target (e.g, 2400 events for an 8-plex assay) in gate A+B (do not use total events) so that any events that fall out of gate A+B will not be recorded.

Figure 29

2.3.3   Save the document one last time and this will be the template for future experiments. The flow cytometer is now ready for sample acquisition.

         The template should be saved in a desired location where it is easily accessible from the computer connected to the flow cytometer.

3. Open a Saved Template for Sample Acquisition
To use an existing template for sample acquisition, follow the instructions below.

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3.1. From the CytoExpert Software home screen, go to Cytometer >> Detector configuration. From the popup window, select the detector configuration that was used to create the template >>set as current >>OK (This is important, as any new experiment must use the same detector settings as that for the template).

3.2 From the CytoExpert software home screen, click New experiment from template. Alternatively, go to File, click New Experiment from template.

3.3 From the popup window, click browse to create a folder for data storage and to select the saved template (a file with ".xitm" extension)

3.4 From the Cytometer tab, open Acq. setting.

3.5 Run Setup Beads 3: Raw Beads to verify the following settings. Adjust the gains of each channel as necessary.

A beads and B Beads are in the predefined gate and FSC/SSC readings are $\geq 20 \times 10^4$.

PE fluorescence intensities of all beads are between 10^2-10^3 (between ~200-800)

APC (or AF647) fluorescence intensities of all beads are between 10^3-3×10^5.

3.6 Run Setup Beads 2: PE Beads to verify that PE fluorescence intensities of the PE positive beads are between 2.5×10^5-3×10^5. Adjust the gain of the PE channel as necessary.

3.7 Refer to section 2.3.2 to set events to record if the new assay has a different plex size (number of targets) as the template.

3.8 The flow cytometer is now ready for sample acquisition.