

Sony SH800 setup guide for LEGENDplex[™] assays

Before Starting the Assay:

- For LEGENDplex[™] analysis, the SH800 cytometer should be equipped with **both 488nm and 638nm lasers**. Check the configuration of your cytometer before starting the assay.
- Check which FL channels are assigned to **PE and APC detection**.
- **Extra C0 and C7 standards** are essential for optimizing PMT voltages (see 2.2).

Instrument Setup:

1. Start up the Instrument

Start up the SH800 instrument and run automatic calibration, following the manufacturer's instructions.

2. Create a New Experiment for Data Acquisition

2.1 Set up FSC-A / BSC-A Density Plot

2.1.1

After successfully completing the automatic calibration, click **[OK]** to proceed to the **[Create Experiment]** window. In the event that the **[Create Experiment]** window is not displayed, click **[New]** on the ribbon within the **[Experiment]** tab to open the **[Create Experiment]** window.

2.1.2

Select **[Blank Experiment]** to create a new experiment.

Note: If a specific template for a LEGENDplex[™] experiment has previously been added to **[My Template]** as is shown in 2.3.3., select the corresponding template and click **[Create New Experiment]**.

Select **[Start acquiring first tube]** and click **[OK]** in the **[New Experiment Startup Procedure]** window which pops up after clicking **[Create New Experiment]**. Then proceed to 2.3.1.

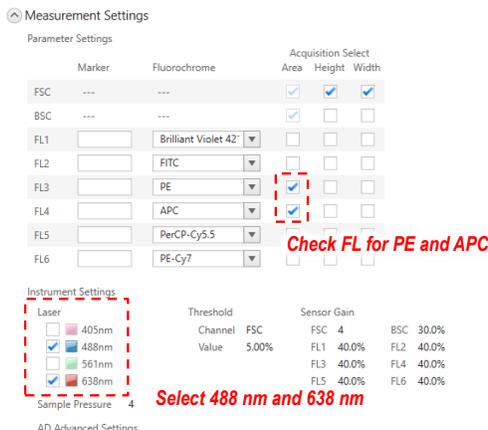


Fig. 1 Measurement Settings
(4 laser-equipped instrument)

2.1.3

Fill in **[Experiment Information]** and **[Sample Group Information]** as necessary. Check measurement settings as described in **Fig. 1** and click **[Create New Experiment]**. Identify the FL channels which allow detection of PE and APC, as these can vary depending on the laser/filter configuration of each cytometer. The other FL channels are not required. Turn on the 488 nm and 638 nm lasers, both of which are necessary for the assay. Select **[Start acquiring first tube]** and click **[OK]** in the **[New Experiment Startup Procedure]** window that pops up after clicking **[Create New Experiment]**.

2.1.4

The first tube will be assigned and a default FSC-A / BSC-A density plot displayed on the worksheet after creating a new experiment.

Remove Gate-A (initially set on the FSC-A / BSC-A density plot), and left-click anywhere within FSC-A / BSC-A density plot to enable the gate selection tool on the ribbon.

Choose polygon gate on the ribbon to create two separate gates on FSC-A / BSC-A density plot as is shown in **Fig. 2**. The left gate is for the smaller A beads and the other for the larger B beads. The location and the shape of these gates will be adjusted later in this protocol (see 2.2.4).

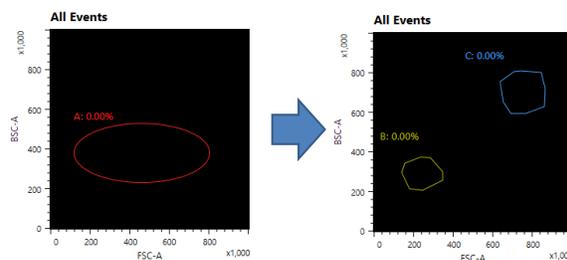


Fig. 2 Gate Settings

2.1.5.

Double-click inside both of the gates on the FSC-A / BSC-A density plot to create child plots for each respective parent gate.

Change the title on Y-axis to “APC-A compensated” and X-axis to “PE-A compensated” by left-clicking the axis titles on both of the child plots.

Full-ranged logarithmic scale should be utilized for both axes on the APC-A / PE-A density plots. Click **[Default Adjust]** on the ribbon and choose **[6 decades]** to permit the display of the full axis range (**Fig. 3**).

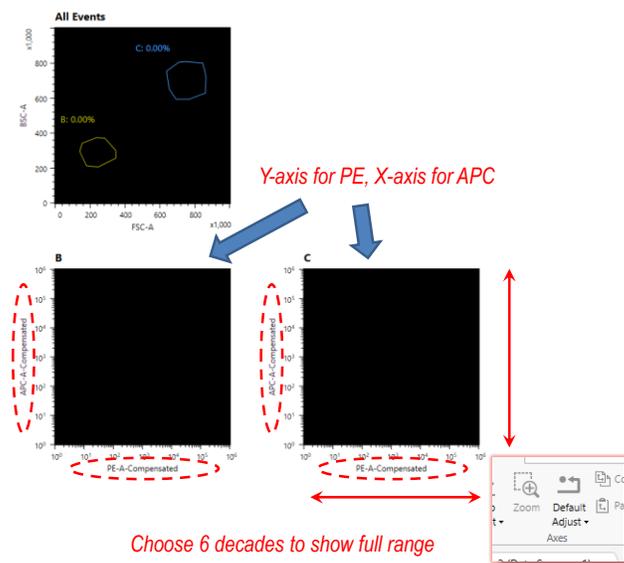


Fig. 3 Scale Adjustment

2.2 Optimization of PMT Voltages and Compensation

For PMT voltage adjustment and compensation, C0 and C7 are to be utilized. **Prepare extra C0 and C7 separately from the ones to be involved in standards**, following the instruction in “Standard Preparation” in your LEGENDplex[™] assay manual.

2.2.1

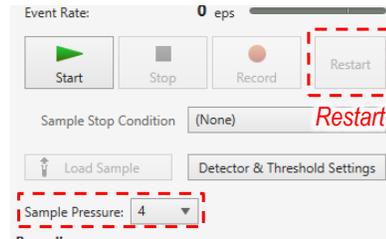
Vortex the vial of Raw Beads for 30 seconds to re-suspend the beads. Sufficient vortex mixing is important to prevent aggregation of the beads.

2.2.2

Transfer 400 µL of Raw Beads to a fresh 1.5 mL tube.

2.2.3

Set the sample pressure to 4 (**Fig. 4**). Place the beads solution in the sample loader and click **[Start]** to load the tube. Click **[Restart]** 10 seconds after the events appear on the plot.



Sample pressure = 4

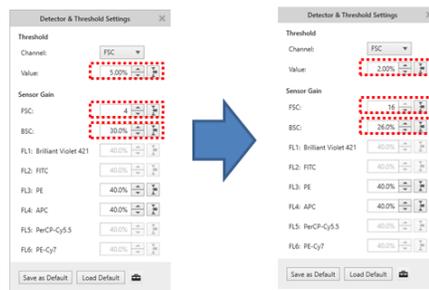
Fig. 4 Data Acquisition

2.2.4

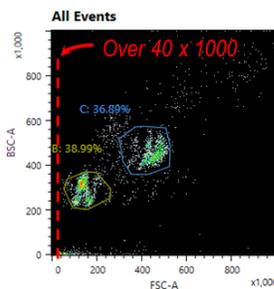
Click **[Detector and Threshold Settings]** and optimize the gain of FSC-A and BSC-A detectors so that the both smaller and larger beads are completely displayed within the FSC-A / BSC-A density plot.

Here, typical FSC gain should be 16. FSC-A value of all dots derived from the smaller beads should be over 40 x 1000. Set the **[Threshold Value]** to 2.0%.

Adjust the size and position of the pre-arranged gates in 2.1.4., so that both bead populations fall into their respective gates (**Fig. 5**).



Value to 2%, Maximize FSC, and adjust BSC



Move gates to the right position.

Fig. 5 FSC, BSC Adjustment

2.2.5

Two populations (APC-high and APC-low populations) should appear on both of the APC-A compensated / PE-A compensated density plots corresponding to smaller and larger beads, respectively. Adjust APC sensor gain so that the APC fluorescence intensities of all beads range between 1×10^3 and 2×10^5 (**Fig. 6**).

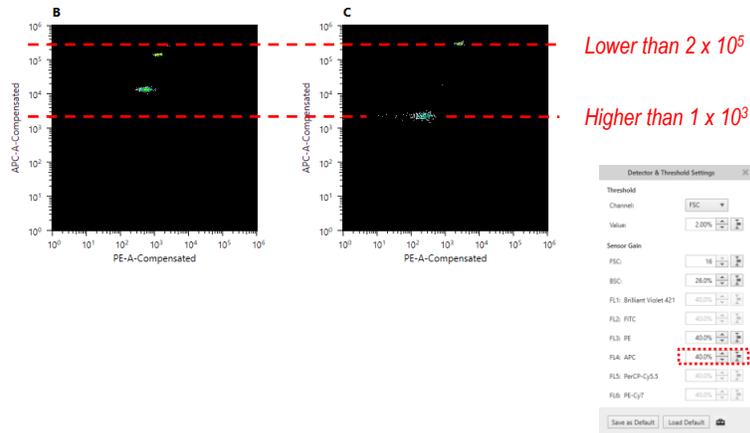


Fig. 6 FL-APC Adjustment

2.2.6

Transfer 100 μ L of extra C0 and C7 standards into a single 1.5mL tube. Close the tube lid and mix the beads solution by flipping over the tube three times.

2.2.7

Set the sample pressure to 4. Place the C0 + C7 beads solution in the sample loader and click [Start] to load the tube. Click [**Restart**] 10 seconds after the events appear on the plot.

2.2.8

Adjust PE sensor gain so that the highest PE intensity among all C7 populations reaches 5×10^5 . After this adjustment is finished, unload the tube (**Fig. 7**, next page).

2.2.9

Left-click once on the APC-A compensated / PE-A compensated density plots of smaller beads.

Click **[Manual Compensation]** on the ribbon within **[Compensation]** tab.

Compensate until the APC fluorescence intensity of each C7 bead ID is matched to its corresponding C0 bead ID.

After compensation has been finished, click **[Manual Compensation]** on the ribbon again to release manual compensation function (**Fig. 8**).

Note: Defining an optimal sensor gain for the PE channel which gives the widest dynamic range is critical to obtain the best results.

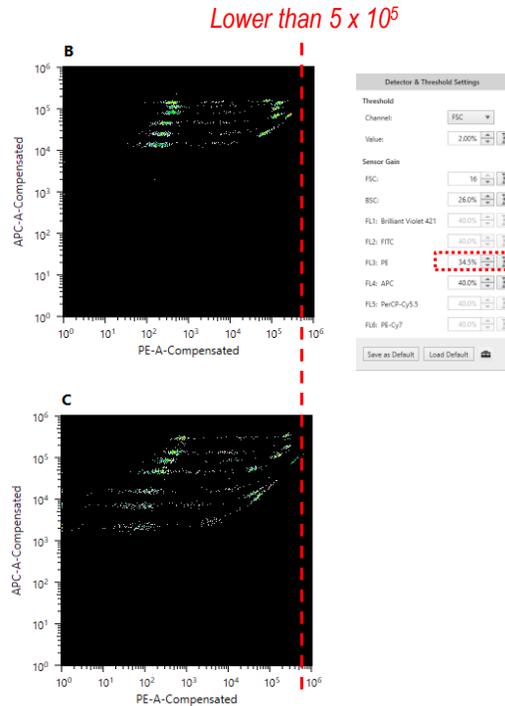
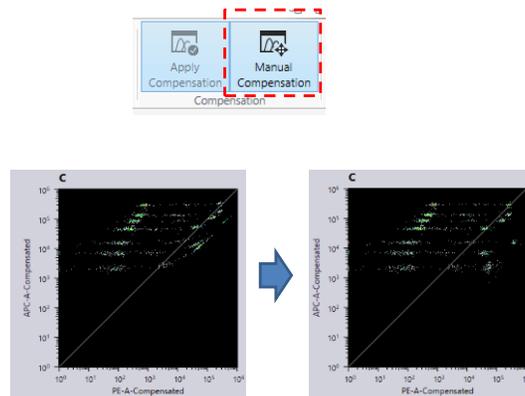


Fig. 7 FL-PE Adjustment

If the voltage is too low, then the low end of the standard curve may plateau due to low signal-to-noise ratio. If the voltage is set too high, the high end of the standard curve may plateau due to signal saturation.



Compensate manually on the APC / PE plot.

Fig. 8 Manual Compensation

2.3 Gate out Debris and Set Number of Events to Record

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

2.3.1

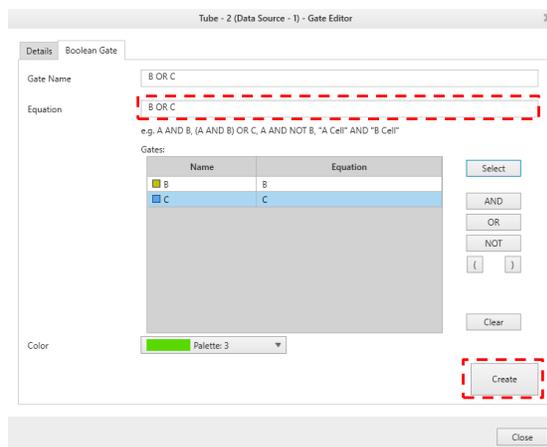
Left-click on the **[Gates and Statistics]** table, and then click **[Edit Gate]** on the ribbon.

Click **[Boolean Gate]** tab in **[Gate Editor]** to create a new gate.

Fill in **[Gate Name]** with an arbitrary name

Fill in **[Equation]** with the following: (“gate name” for smaller beads) OR (“gate name” for larger beads).

For example, if the gates for the smaller and larger beads are “B” and “C” respectively, the equation of the new gate should be “B OR C” (**Fig. 9**).



*Equation ⇒ “B OR C”
If B is for smaller bead
and C is for larger cell;*

Click on “Create”

Fig. 9 Boolean Gate

2.3.2

Select **[Gated Event Count]** as stop condition. Choose the “new gate” created above and set the stop count to 300 events per target (e.g. 2,400 events for an 8-plex assay, 3,900 events for an 13-plex assay). Set **[Sample Stop Condition]** to **[Recording]**.

2.3.3

Right-click once on the corresponding “Experiment” and choose **[Save as Template]** (Fig. 10). The optimized settings and gates for LEGENDplex[™] are saved and can be used the next time you run the assay.

Note: The optimal instrumental settings will be subtly different after each optical alignment. It is strongly recommended users optimize all settings for each new LEGENDplex[™] experiment.

2.3.4

The flow cytometer is now ready for sample analysis.

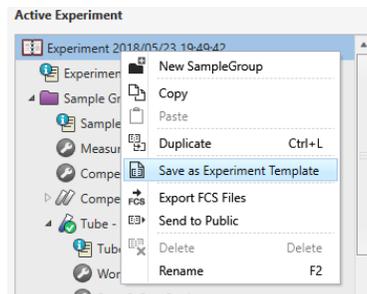


Fig. 10 Template Saving