abcam

Setup protocol for Firefly® particles

On BD LSR II and LSRFortessa cytometers with single tube loader function

Version 1 – Last Updated May 2016

abcam

Setup protocol for Firefly® particles

Contents

This protocol contains instructions for setting up a BD LSR II or LSRFortessa in single Tube Loader function for use in acquiring Firefly® multiplex particles. Some cytometers may not be able to resolve the particles to a sufficient level for analysis. The Cytometer Setup Particles used in this assay are designed to help identify properly resolving machines for Firefly® multiplex assays. If you have questions about compatibility, contact our Technical Support team at technical@abcam.com.

If you will be using a different system, consult the appropriate protocol in our flow cytometry protocols for multiplex assays page, or contact our Technical Support team at technical@abcam.com.

Please read this protocol carefully before cytometer operation. Failure to properly set up a system may result in unusable data, loss of product or system damage. For more detailed instructions on system operation please consult the BD digital cytometer operation manual and consult your machine's designated operating technician.

Cytometer requirements

- BD digital cytometer running FACSDiva 6.1.3 acquisition program, or later version.
- One of the either configuration:
    - 488 nm (Blue Laser) excitation for "Green", "Yellow", "Red" fluorescence [**recommended**]
        - Green Filter (e.g. FITC, 530/30 Bandpass filter).
        - Yellow Filter (e.g. PE, 575/25 Bandpass filter).
        - Red Filter (e.g. PE-Cy5, 685/35 Bandpass filter; or PerCP-Cy5.5, 695/40 Bandpass Filter).

      OR

    - 488 nm (Blue Laser) excitation for "Green" and "Red" fluorescence; and 561 nm (Yellow/Green Laser) excitation for "Yellow" fluorescence.

Protocol requirements

- Provided Components:
    - Cytometer Setup Particles (ab211043)
    - 1X PBS
    - Microdilution Tubes (1.2 mL, polypropylene)

- Required, but not provided:
    - 12 x 75 mm (5 mL) round-bottom polystyrene tubes (Corning, #352008)
    - Forceps

I. Template file import and example file demonstration

1. Confirm what version of FACSDiva software your machine uses and download the appropriate acquisition Firefly template file from www.abcam.com/FireflyCytometry.

    a. ***If running FACSDiva version 6***, download one of the "Firefly Diva 6" zip files (PerCP, PerCP-Cy5_5 or PE-Cy5 version depending on your machine's detector configuration).

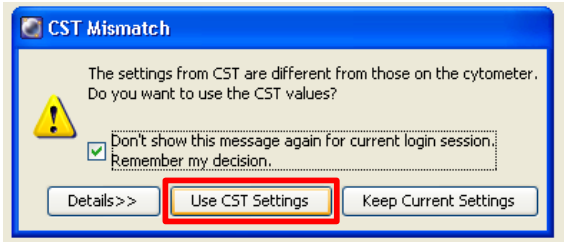
    b. ***If running FACSDiva version 7 or version 8***, download the "Firefly Diva 7" zip file.

2. Transfer the appropriate template file to a USB drive to import it onto the computer driving the cytometer.

3. Start up the BD cytometer and allow at least 15 minutes for laser warmup.

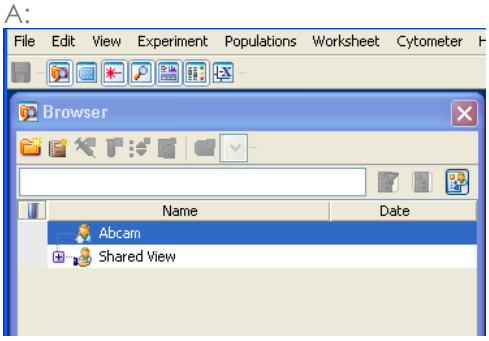
4. Launch the FACSDiva acquisition software. Be sure to have the cytometer administrator perform all requisite system start up quality control and cleaning functions (Cytometer Setup and Tracking protocol, CST) to ensure normal cytometer performance.

5. Log into your FACSDiva profile. If a CST Mismatch warning shows, check "Don't show this message" and then click the "**Use CST Settings**" button.

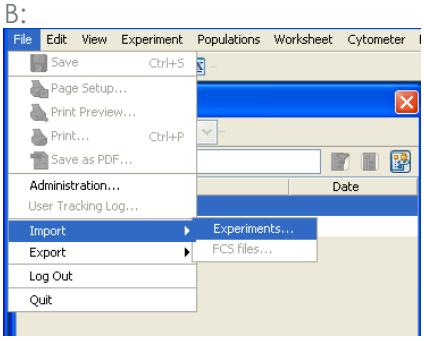


6. Highlight your user profile icon in the "**Browser**" window (A). Click the "**File Menu**", select "**Import**", then select "**Experiments**" (B). Locate the Firefly Diva template file on your USB drive and select the file for import.

A:

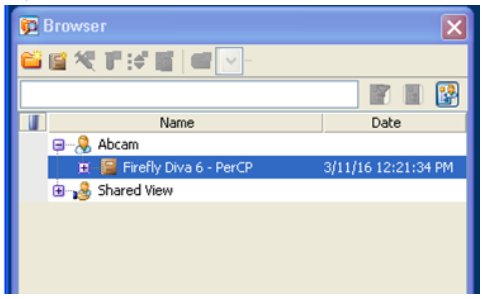


B:

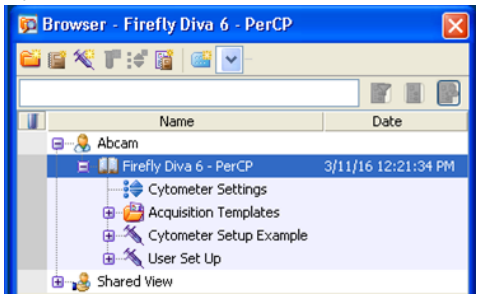


7. Once the template is loaded, **double-click** on the closed template file ( , A) to "open/activate" it ( , B). A "**Configuration Mismatch**" warning may come up (C). Select "**Continue**" on the warning if it appears.

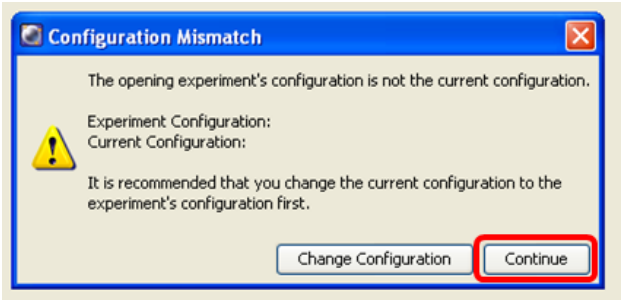
A:



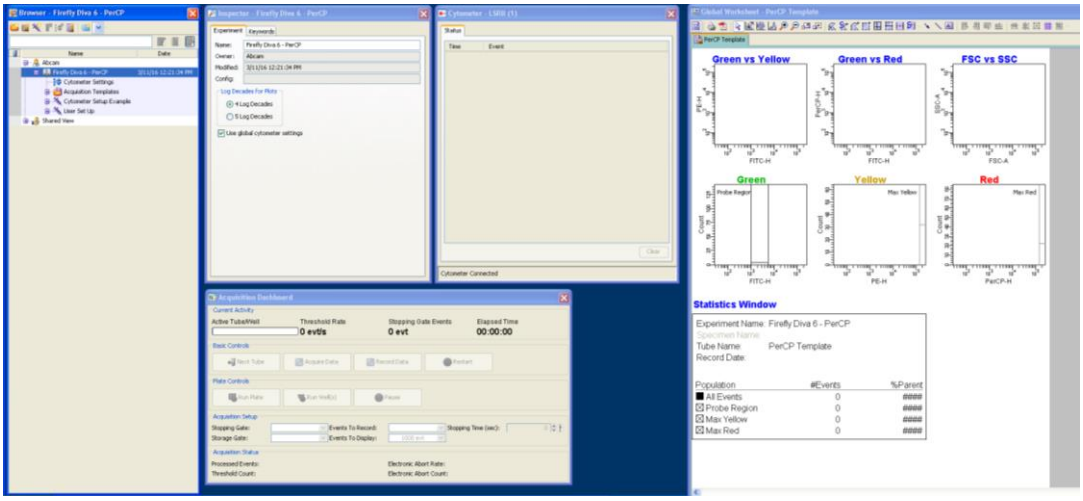
B:



C:

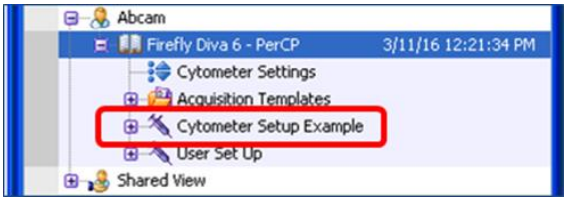


8. Once the experiment is opened, the window should appear like the following.

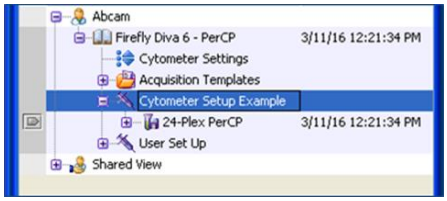


9. In the open experiment, locate the "**Cytometer Setup Example**" specimen (A) and expand by clicking the "+" symbol to expose the "**24-Plex**" sample data (B). ***This is an embedded FCS file that is provided for demonstration purposes only for the users to familiarize themselves with setting proper detector voltages.***

A:

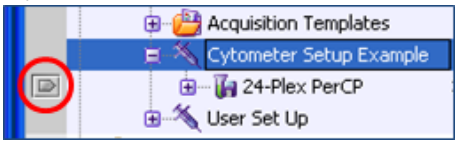


B:

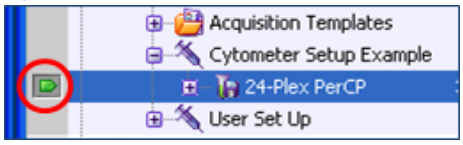


10. Display the data by clicking the Tube Selector icon ( ) to the left of the 24-Plex sample data (A). The Tube Selector icon will turn green ( ) when the data are selected (B).

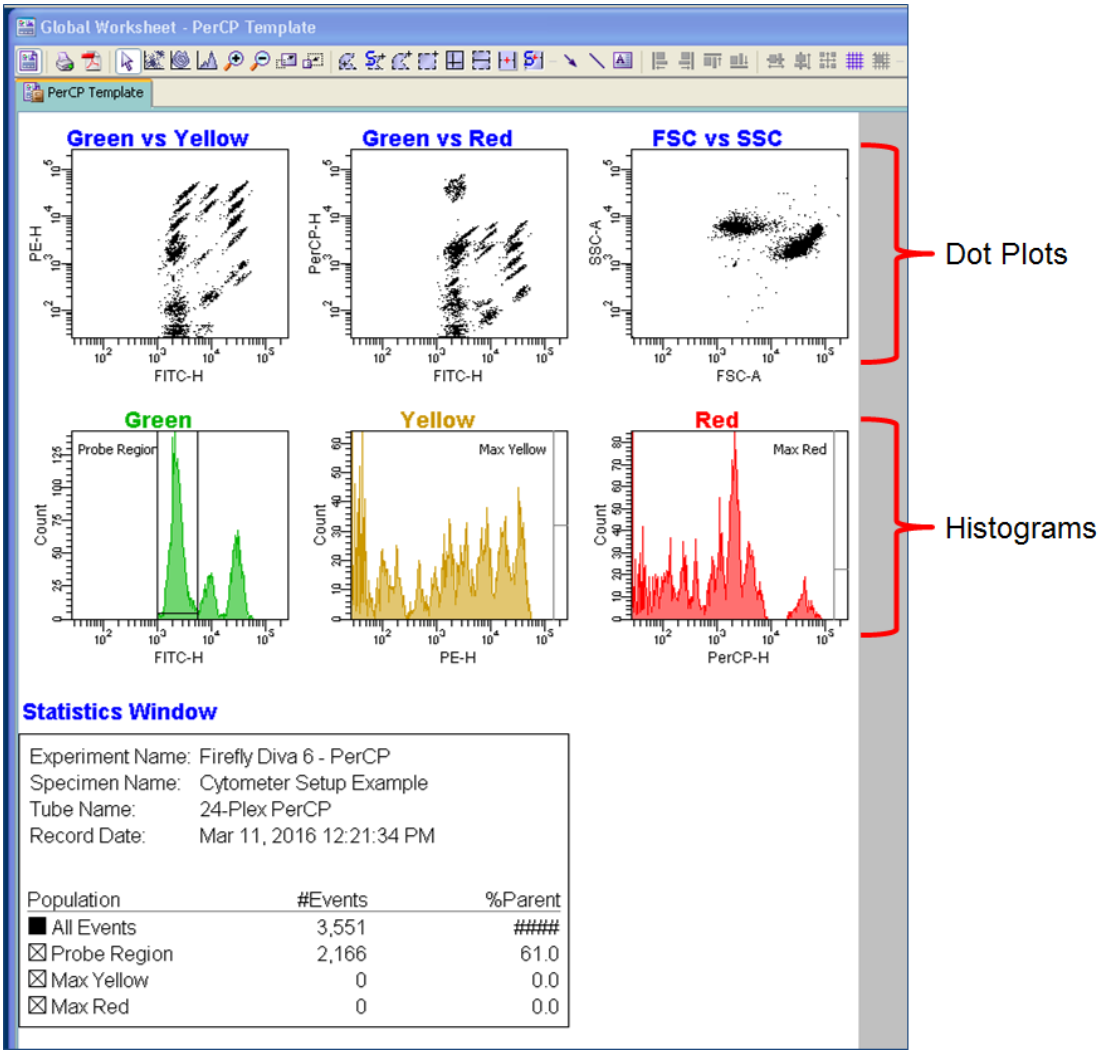
A:



B:



11. The Example data file will now display in the "**Global Worksheet**" page and should appear as below with 3 dot plots (labeled "**Green vs Yellow**", "**Green vs Red**" and "**FSC vs SSC**") and 3 colored histograms (labeled "**Green**", "**Yellow**", and "**Red**").



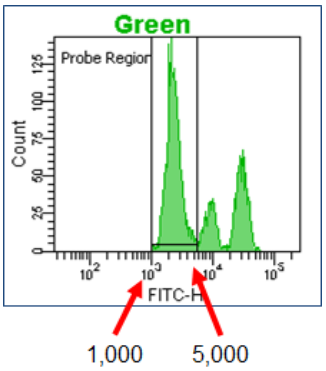
12. Locate the "**Statistics Window**" below the colored histograms. Observe that the line labeled "**Probe Region**" has a "**%Parent**" column value that is about 60%.

Statistics Window

Experiment Name: Firefly Diva 6 - PerCP
Specimen Name: Cytometer Setup Example
Tube Name: 24-Plex PerCP
Record Date: Mar 11, 2016 12:21:34 PM

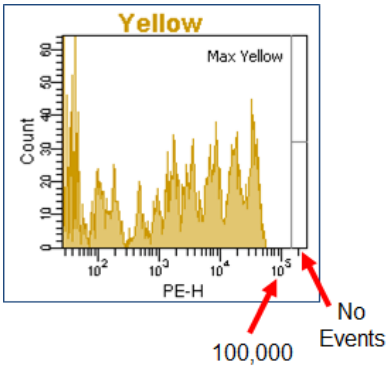
Population	#Events	%Parent
■ All Events	3,551	####
☒ Probe Region	2,166	61.0
☒ Max Yellow	0	0.0
☒ Max Red	0	0.0

13. Locate the "**Probe Region**" interval gate on the "**Green**" plot spans FITC-H ("Green") values of approximately 1,000 (10^3 marker) to 5,000. This is the ideal FITC-H positioning and distribution of the furthest left FITC peak (i.e. the Probe Region of the Firefly particles). **Three distinct Green peaks** should be visible on this plot.



14. Locate the "**Yellow**" histogram. Observe that no events appear in the "**Max Yellow**" interval gate located to the right of the 100,000 (i.e. 10^5) marker of the PE-H scale (A). In the "**Statistics Window**", the "Max Yellow" line should display values of "**0**" under both "**#Events**" and "**%Parent**" columns (B).

A:



B:

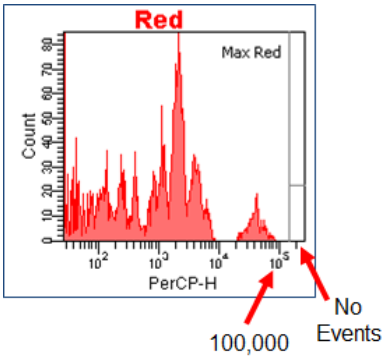
Statistics Window

Experiment Name: Firefly Diva 6 - PerCP
Specimen Name: Cytometer Setup Example
Tube Name: 24-Plex PerCP
Record Date: Mar 11, 2016 12:21:34 PM

Population	#Events	%Parent
■ All Events	3,551	####
☒ Probe Region	2,166	61.0
☒ Max Yellow	0	0.0
☒ Max Red	0	0.0

15. Locate the "**Red**" histogram. Observe that no events appear in the "**Max Red**" interval gate located to the right of the 100,000 (i.e. 10^5) marker of the PE-Cy5-H (or PerCP-H, or PerCP-Cy5.5-H) scale (A). In the "**Statistics Window**", the "Max Red" line should display values of "**0**" under both "**#Events**" and "**%Parent**" columns (B).

A:



B:

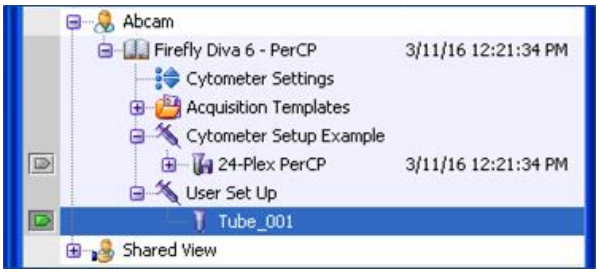
Statistics Window

Experiment Name: Firefly Diva 6 - PerCP
Specimen Name: Cytometer Setup Example
Tube Name: 24-Plex PerCP
Record Date: Mar 11, 2016 12:21:34 PM

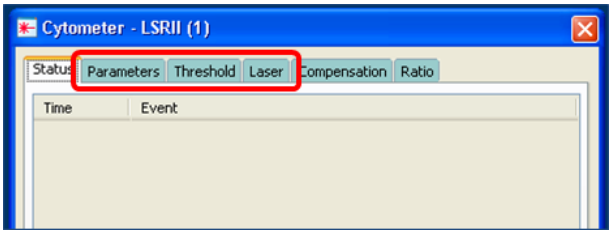
Population	#Events	%Parent
■ All Events	3,551	####
☒ Probe Region	2,166	61.0
☒ Max Yellow	0	0.0
☒ Max Red	0	0.0

II. Cytometer setup particle acquisition for voltage optimization

16. In the "Browser Window", locate the "**User Set Up**" icon and expand the "+" button to reveal the "**Tube_001**" sample. Click the Tube Selector icon on left to make it green to select "**Tube_001**" for settings adjustments. This sample contains no saved data, so the Global Worksheet will now have empty data plots.

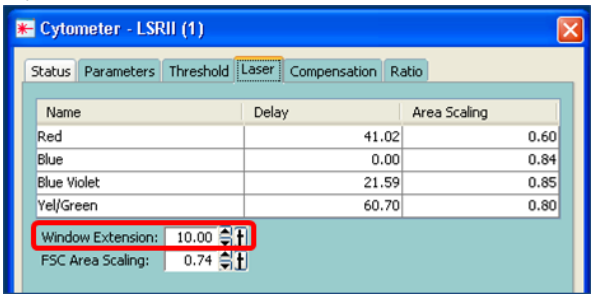


17. Locate the "**Cytometer**" control window that contains the "**Parameters**", "**Threshold**", and "**Laser**" tabs.

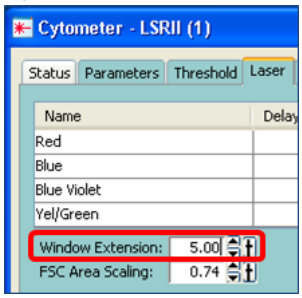


18. Select the "**Laser**" tab and locate the "**Window Extension**" parameter that is set to its standard "**10.00**" value (A). Change the Window Extension value to be "**5.00**" (B). *Note: If the Window Extension adjustment window is not available to you in this tab, contact your machine's designated Administrator to enable Window Extension editing.*

A:



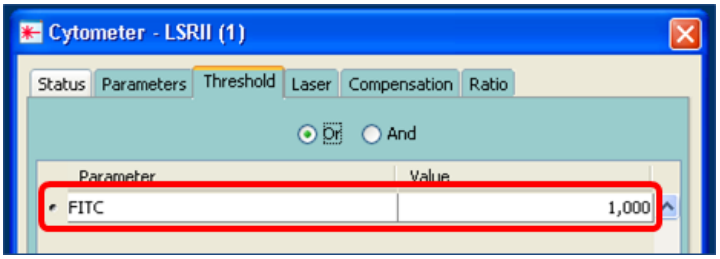
B:



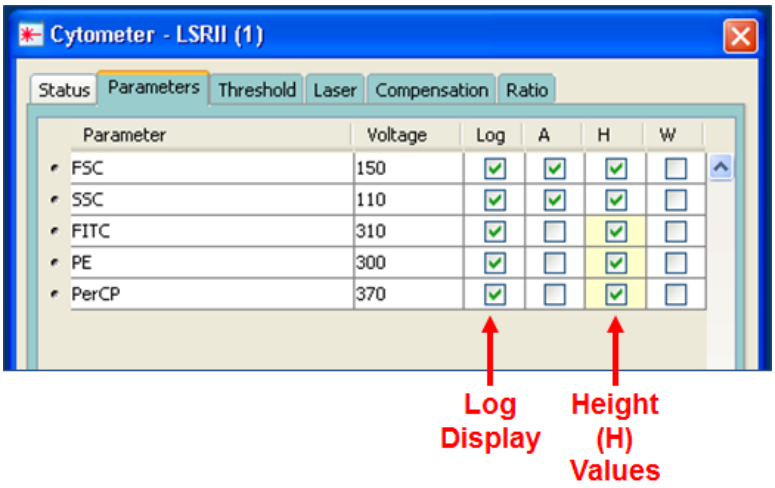
****** A Window Extension value of 5.00 is critical to ensure Firefly® particle compatibility on LSR II and LSRFortessa cytometers. ******

****** Failure to maintain the 5.00 Window Extension value between sample acquisitions will cause an irrecoverable loss of data. ******

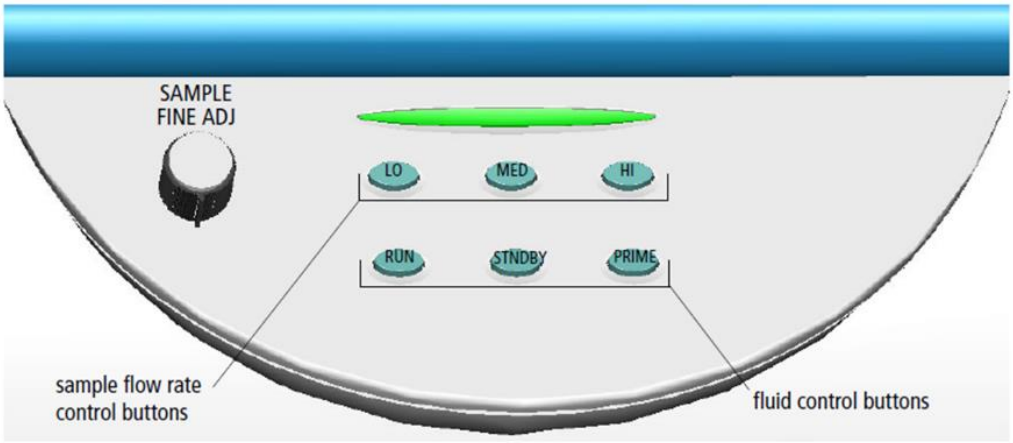
19. In the "**Cytometer**" control window, select the "**Threshold**" tab. Ensure that the displayed parameter is "**FITC**" and the Value is "**1,000**".



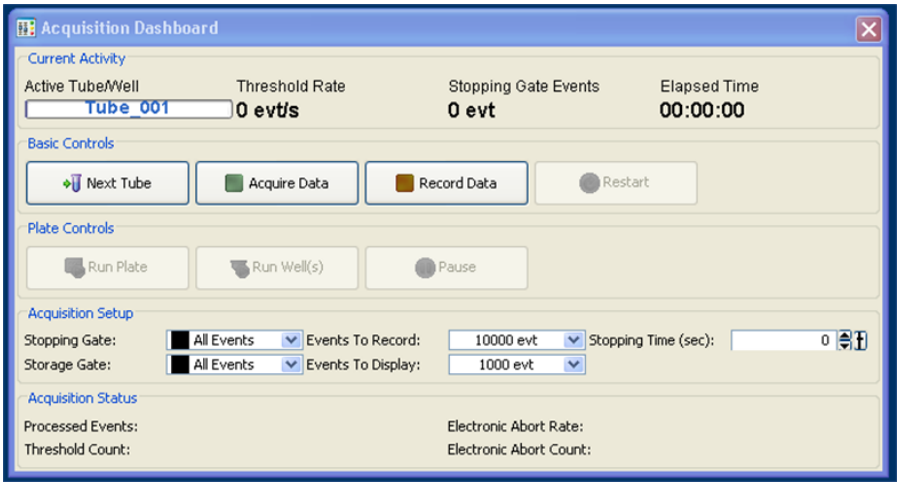
20. In the "**Cytometer**" control window, select the "**Parameters**" tab. The window should appear similar to the following where the fluorescent detector parameters and their corresponding voltage settings are displayed. Ensure that all parameters are set to "**Log Display**" (checked "Log" box). Ensure that the fluorescent parameters (e.g. FITC, PE, PE-Cy5) are set to record "**Height**" data (checked "H" box) and have no check in the "Area" box (clear "A" box).



21. Before placing the sample tube on the machine, verify the fluidics controls on the front of the machine. The machine should be in "**Standby**" mode ("**STNDBY**" button, lit orange when engaged). Locate the "**Sample Fine Adjust**" knob on the left side of the panel. **Rotate the knob Clockwise** until it stops; this is now set on the highest flow rate for Fine Adjust. Locate the **Medium** speed flow rate button ("**MED**" button) and press to select.



22. Locate the "**Acquisition Dashboard**" control panel where the Basic Control buttons of "**Next Tube**", "**Acquire Data**" and "**Record Data**" are located.

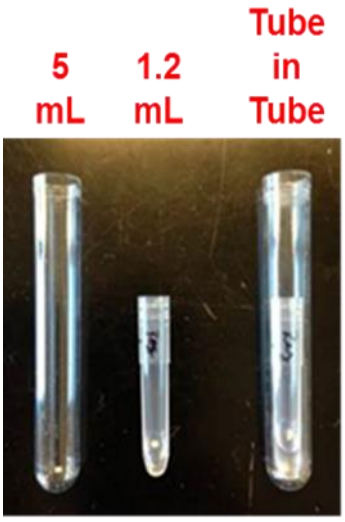


****** Please familiarize yourself with the following Steps 23-30 BEFORE placing samples on the cytometer. ******

****** Please move quickly through steps 23-30 to prevent loss of test samples. ******

****** If you are unfamiliar with the operation of a BD flow cytometer, please consult your machine's designated operating technician. ******

23. Prepare a setup sample by pipetting **200 µL of 1X PBS** into a **1.2 mL Microdilution Tube**. Vortex the tube of **Cytometer Setup Particles** (ab211043) and pipet **35 µL of particles** into the PBS in the microdilution tube. Place the entire tube within a 5 mL polystyrene tube to enable loading the sample onto the Sample Injection Port (SIP) of the cytometer. The cytometer SIP should already have a 5 mL tube containing DI Water on it.



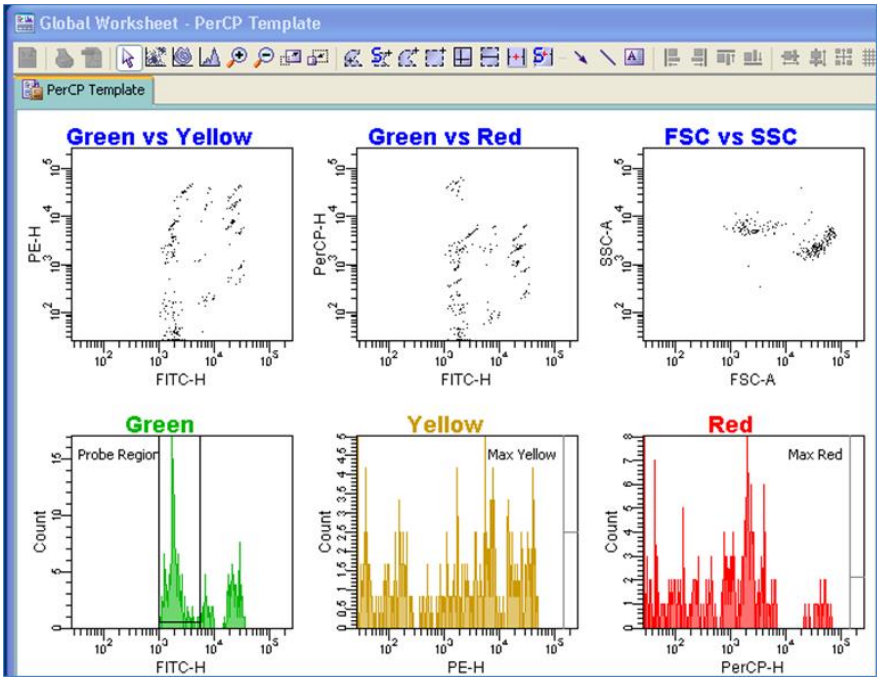
24. Remove the 5 mL tube containing DI Water from the cytometer and place the tube of Firefly particles on the SIP in its place. Switch the fluidics control to "**RUN**" using the button on the front of the machine. The "**RUN**" button should be lit green when engaged.

25. In the "**Acquisition Dashboard**" control panel, click the "**Acquire Data**" button to begin seeing data events (A). The button will switch to display as "**Stop Acquiring**" and the "**Elapsed Time**" counter will begin counting up (B). Data should begin appearing in the "Global Worksheet" window (C).

A:

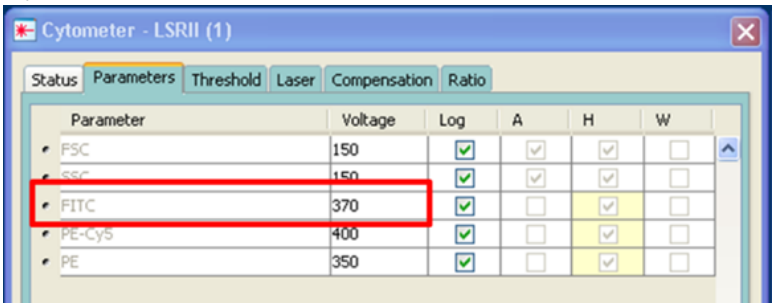
B:

C:

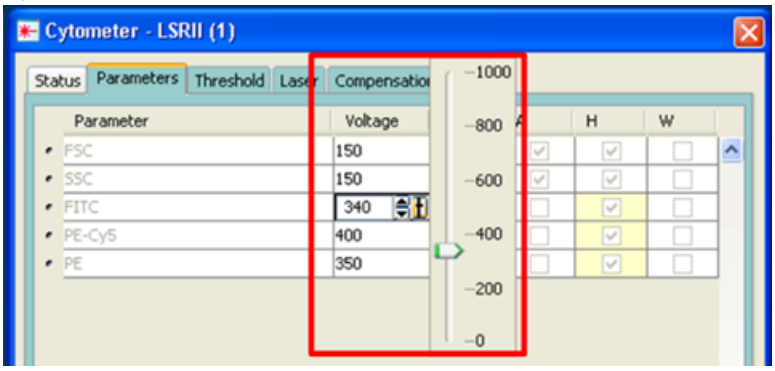


26. Adjust the voltages of the all the channels as needed to get your data to appear similar to the example FACS plots provided in Steps #11-15 of this protocol. For example, to raise or lower the FITC voltage to get the Probe Region green fluorescence in the provided gate on the "**Green**" histogram, click on the "**Parameters**" tab within the "**Cytometer**" control panel. Locate the FITC parameter line and click the "**Voltage**" window for that line (e.g. 370 value) (A). The window will become active to enable editing. Click the icon on the right side of the Voltage column to expand the Voltage Slider control (values 0 to 1,000). Type in desired voltages in the window or use the slider to select higher or lower values (B).

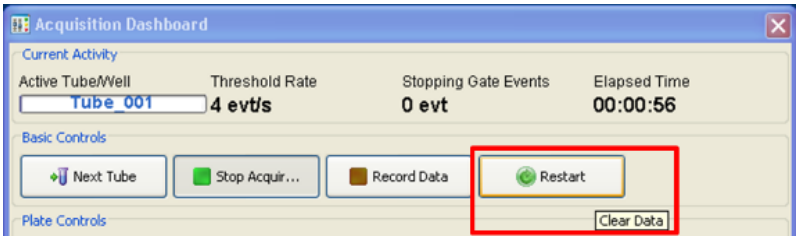
A:



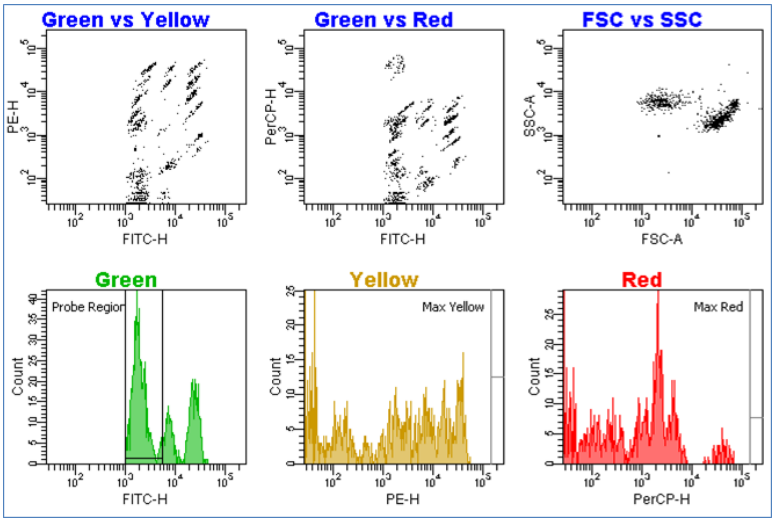
B:



27. When a new voltage is selected for viewing, hit the "**Restart**" button in the Acquisition Dashboard to clear the previous voltage data to allow new voltage data to be viewed in the Global Worksheet.



28. Observe the new data collected on the parameter voltages you have adjusted. Repeat any necessary changes until all 3 fluorescent parameters and the 2 non-fluorescent parameters (i.e. "**FSC**" and "**SSC**") are on scale (i.e. particle events should not exceed 10^5 values on FACS plot scales). There should only be **one peak visible in the "Probe Region" interval gate of the "FITC-H" histogram**.



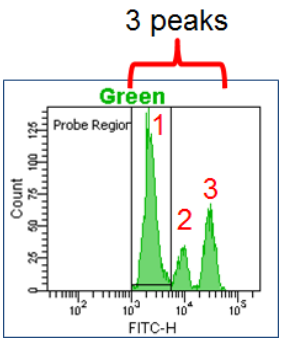
29. After adjusting the voltages such that all parameters are on scale in the Acquisition mode, data must now be Recorded to enable downstream analysis. Locate the "**Record Data**" button in the "Acquisition Dashboard" and click the button to begin saving data (A). When engaged, the button icon will turn orange and display a "**Stop Recording**" command (B). "**Stopping Gate Events**" counts will also begin counting up as new data points are stored.

A:

B:

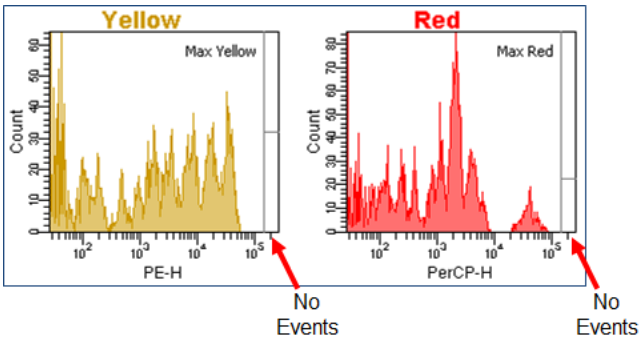
30. After about 500 events have been acquired, hit the "**Stop Recording**" button in the Acquisition Dashboard then hit the "**Stop Acquiring**" button. Remove the sample from the SIP and place the tube containing DI Water back on. Allow the cytometer to continue in "**RUN**" mode using the water. Check to make sure that all data are still below the 10^5 value for all of the displayed histograms.

31. Locate the "**Green**" histogram plot. Ensure that you can distinguish 3 distinct peaks and that the "**Probe Region**" interval gate spans only 1 peak.

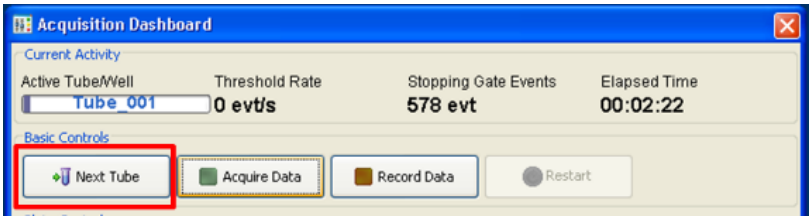


32. Locate the "**Statistics Window**" below the colored histograms. Observe that the line labeled "**Probe Region**" has a "**%Parent**" column value that is about 60%.

33. Locate the "**Yellow**" and "**Red**" histograms. Observe that no events appear in the "**Max Yellow**" and "**Max Red**" interval gates located to the right of the 100,000 (i.e. 10^5) markers. In the "**Statistics Window**", the "**Max Yellow**" and "**Max Red**" lines should display values of "**0**" under both "**#Events**" and "**%Parent**" columns.



34. In the Acquisition Dashboard control panel, locate the "**Next Tube**" button and click it. A new sample tube named "**Tube_002**" will appear in the Browser window below the saved sample data of "**Tube_001**."



35. Click the Tube Selector icon of "**Tube_002**" to turn it green ().

36. Prepare a second test sample in a **1.2 mL Microdilution Tube** using **200 µL of the 1X PBS** and, vortexing again the tube of **Cytometer Setup Particles, add 35 µL of particles** to the PBS. Extract the previously acquired 1.2 mL tube from the 5 mL tube using **Forceps** and place the spent tube off to the side.

37. Place the newly prepared sample tube in a 5 mL Tube on the cytometer SIP and hit "**Acquire Data**". Once new events begin showing up, hit the "**Record Data**" button and turn on High Speed fluidics control button ("**HI**" button) on the front of the cytometer to begin the highest speed acquisition possible.

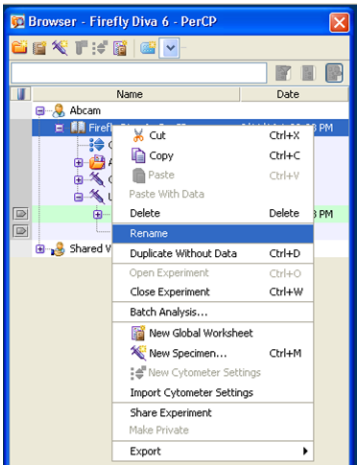
38. Monitor the level of sample left in the tube on the SIP. Continue to record data until almost all the sample has been aspirated from the 1.2 mL tube. Once the sample is nearly gone, hit the "**Stop Recording**" button to conclude data acquisition. Then hit the "**Stop Acquisition**" button.

39. Remove the nearly empty sample from the SIP and replace with the DI Water tube again. Once done with acquiring test samples, proceed with the BD manufacturer recommended cleaning procedures.

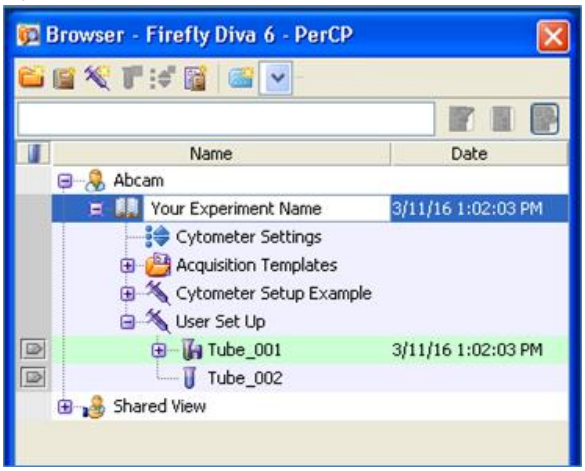
III. Test data export and optimized template storage

40. Rename the Experiment file from its original name (i.e. "Firefly Diva XXXX") to something unique to the day of data acquisition. Rename your experiment by Right-Clicking on the open experiment name (icon) and select the "**Rename**" option (A). Type in a new name for your experiment and hit the "**Enter**" button on your keyboard (B).

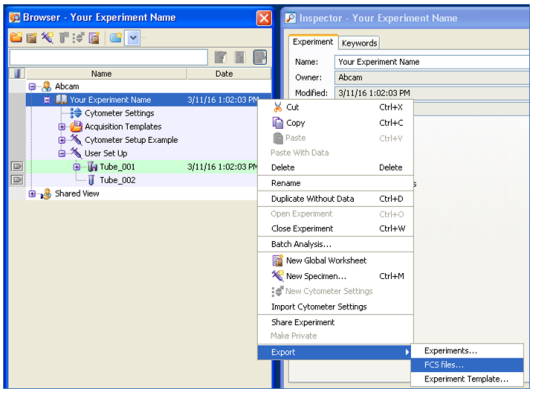
A:



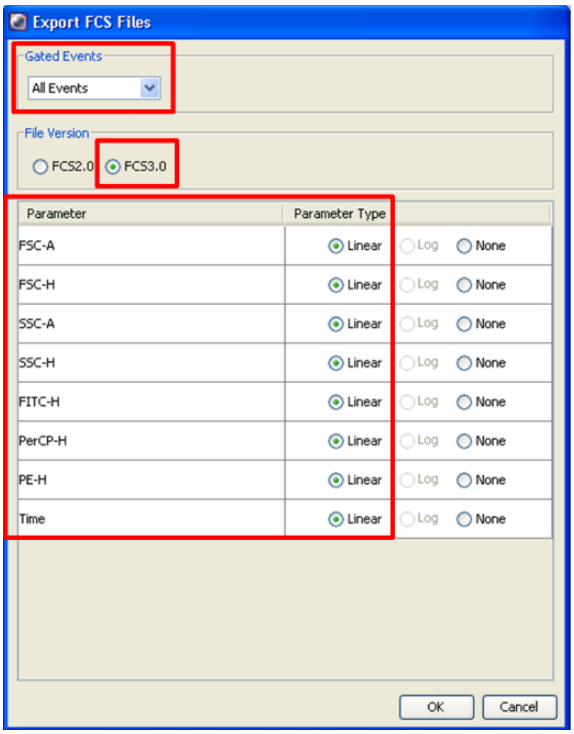
B:



41. Export both the "**Cytometer Setup Example**" (embedded file) and the "**User Set Up**" sample data (your new optimization data) by Right-Clicking on the experiment name (icon), select the "**Export**" option at the bottom and then select "**FCS Files**".

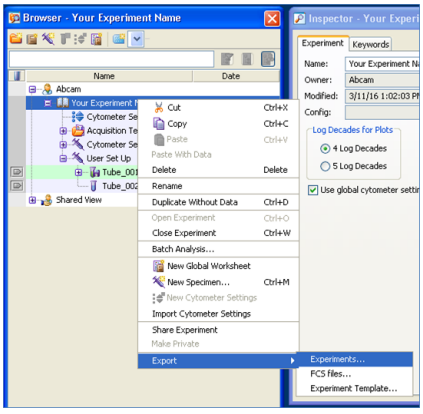


42. You will be prompted with the following option box. Ensure that the FCS files contain "**Gated Events: All Events**", are in "**FCS3.0**" format and all parameters are selected and exported in "**Linear**" format. Hit "**OK**" and then choose the location where the FCS files will be exported to.

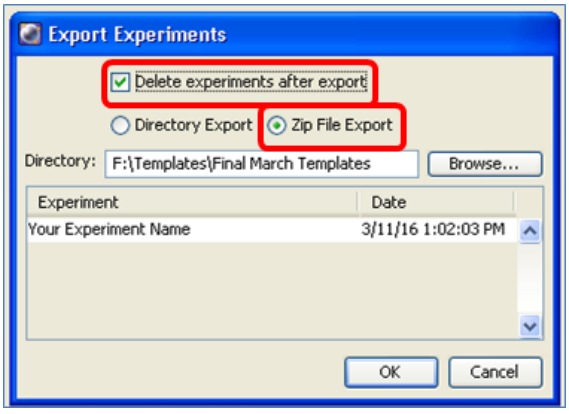


43. Export a copy of today's experiment set to the hard drive so that you can later import the same adjusted settings prior to the next Firefly assay you perform. Right-Click on the experiment name, select the "**Export**" option at the bottom and then select "**Experiments**" (A). You will be prompted to select a location and file type. Select to export in "**Zip File Format**" and select the "**Delete experiments after export**" option (B). Click OK. The experiment will be transferred to the location you specified and will no longer be present in the "**Browser**" window.

A:



B:



IV. Test data analysis

44. To analyze your exported FCS files, download the Firefly® Analysis Workbench onto your personal computer from http://www.abcam.com/kits/firefly-analysis-workbench-software-for-multiplex-mirna-assays.

45. Open the Firefly® Analysis Workbench and load the FCS files saved in Step 42. When prompted, load the PLX file that has been provided for you with your particle mix. Once the data loads, check the color of the well, a Green well indicates a successful setup.

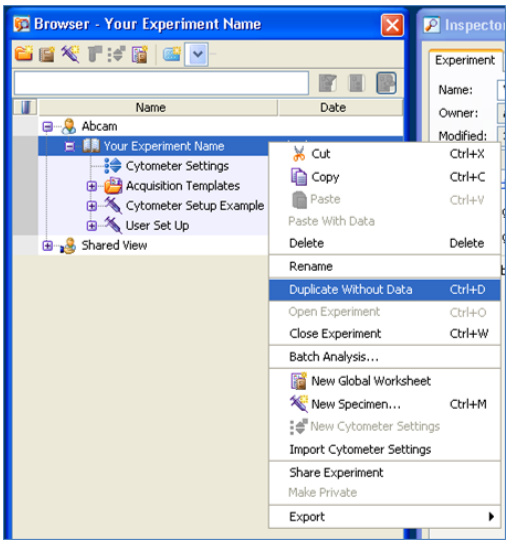
46. Reference your Cytometer Setup Kit instructions for detailed instructions on how to use the Analysis Workbench software to check your data files for proper code recognition and fluorescent reporter level measurement.

If the quality indicator is Red, perform a thorough system clean, and repeat this protocol. If the score remains low, contact our Technical Support team at technical@abcam.com.

V. Assay acquisition after cytometer optimization completion

47. To reimport your optimized cytometer settings in the future, highlight your user profile icon in the "**Browser**" window. Click the "**File Menu**", select "**Import**", then select "**Experiments**". Locate the Zip file on your hard drive that you had previously exported in Step 43 of this protocol.

48. Once imported, double-click on the old experiment to open it. After it opens, you will copy the experiment without transferring the stored FCS files by Right-Clicking on the experiment name (icon) and selecting the "**Duplicate Without Data**" option. A new experiment will be created with the same name plus a "**_001**" identifier at the end of the name.



49. Rename this new "**_001**" experiment with a **new day/experiment-specific title**.

50. Repeat the "**Window Extension 5.00**" and "**Threshold FITC**" checks listed in Steps 16 to 19 of this protocol.

51. Add "**New Specimen**" and/or "**New Tube**" samples and name them specifically for your new experiment.

52. Proceed to acquiring and recording data as listed in Steps 34 to 39 of this protocol. To increase total acquisition speed, use 200 µL of total sample volume per Microdilution Tube. Acquire data with cytometer in "**RUN**" mode using "**HI**" speed.

53. Once all samples have been recorded, export the new FCS files and export the experiment as a ZIP file as instructed in Steps 41 to 43 of this protocol.