Bio-Rad Laboratories, Inc.

Standardizing Assays on ZE5 Cell Analyzers

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Contents

Introduction

Standardization of assays within and across flow cytometer instruments can ensure experiment reproducibility and data reliability. The ZE5 Cell Analyzer Quality Control (QC) process secures instrument operation within Bio-Rad's specifications, but it does not control fluorescence measurements across multiple instruments, or fluorescence measurements on the same instrument over time.

Therefore, to standardize and calibrate fluorescence measurements, this document provides instructions as follows:

- Complete the inter-assay protocol to adjust the ZE5 photomultiplier tube (PMT) voltages using a defined fluorescent bead sample as an external calibration standard.
- Complete the inter-instrument protocol to match the standard across instruments.

Important: These inter-assay and inter-instrument protocols eliminate cytometer settings variability, but they do not control or normalize data variability associated with pipetting, antibody dilution, cell concentration, and so forth.

This document assumes you are familiar with the ZE5 Cell Analyzer and Everest Software. For more information on the steps included herein, see the ZE5 Cell Analyzer and Everest Software User Guide.

Materials

To complete the protocols specified in this document, the following materials must be available:

- ZE5 Cell Analyzer flow cytometer(s)
  - A single instrument for inter-assay standardization
  - Two or more instruments for inter-instrument standardization

  Important: To standardize across flow cytometers, the instruments must have identical laser and filter configurations.
- Spherotech™ Ultra Rainbow Fluorescent Particles, mid-range 10^7 beads/mL 3.5-3.9 µm, 5 mL (Part No. URFP-38-5A)

  This protocol works with any standard fluorescent bead type, but you must ensure the lot is sufficient for all standardization procedures cited herein, and the fluorescence intensity (brightness) is compatible with the assay settings you are using.
- Standard laboratory consumables

  For example, 5 mL round bottom test tubes or microfuge tubes, 96-well plates, pipet tips, pipets, and so forth.
- Phosphate Buffered Saline (PBS) calcium and magnesium-free

Prerequisites

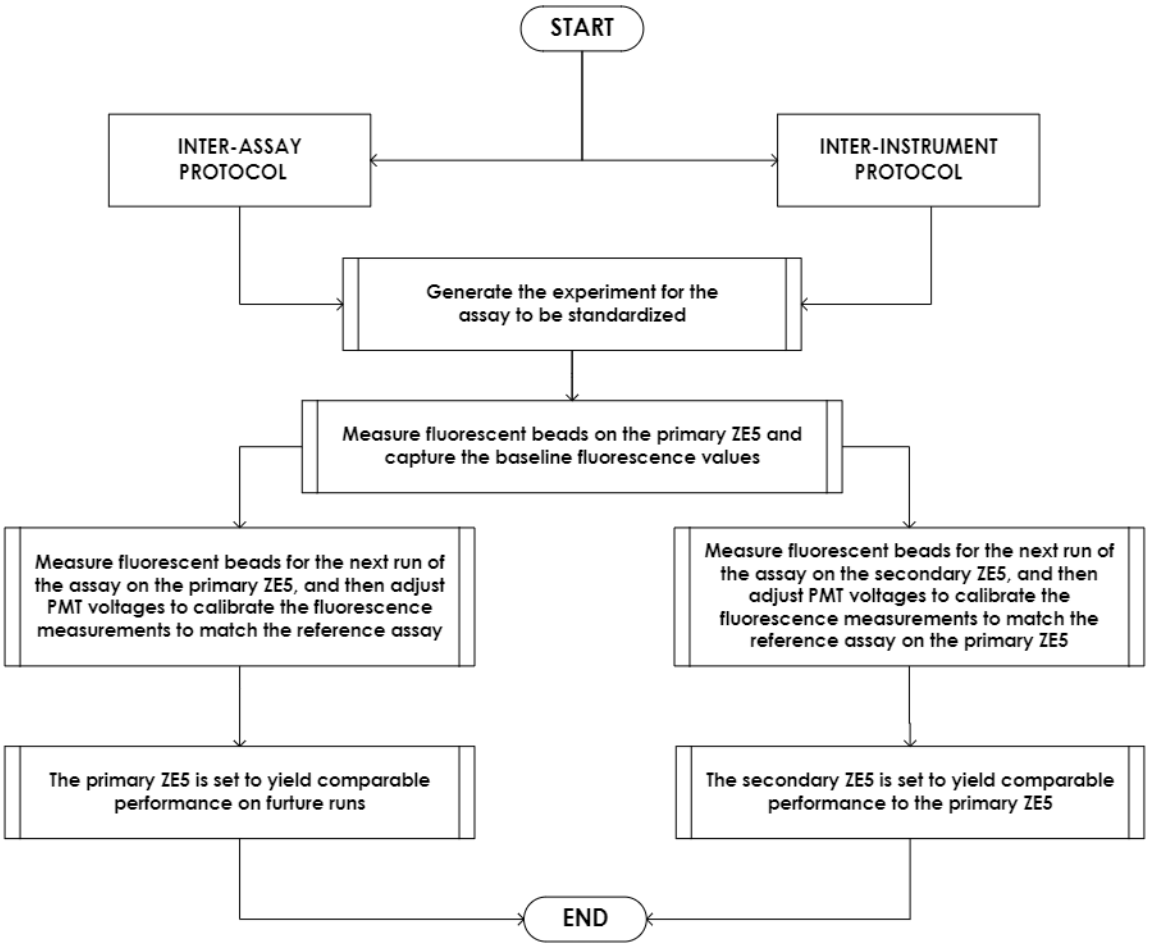
Complete the following prerequisites before you initiate these protocols:

- Ensure the ZE5 instruments being used for assay standardization are in good condition and have passed QC.
- Determine instrument linearity; out-of-range voltage settings might return inaccurate results. For information on linear ranges, refer to the information at the following hyperlink:
  https://www.bio-rad-antibodies.com/detector-linearity

Protocol Process Workflow

Figure 1 illustrates the workflow for each protocol.

Figure 1: Process overview



Inter-Assay Protocol

This protocol contains instructions to standardize the fluorescence measurements of an experiment assay, over time, on the primary ZE5 Cell Analyzer.

The procedures are explained in the following sections:

Section 1: Generating an assay/experiment on the primary instrument (reference assay).

Section 2: Characterizing fluorescence measurements using a standardized bead sample on the primary instrument (baseline fluorescence).

Section 3: Using the same bead sample, adjusting voltages to calibrate fluorescent measurements on the primary instrument for inter-assay standardization on the instrument.

Section 1: Generating the Reference Assay

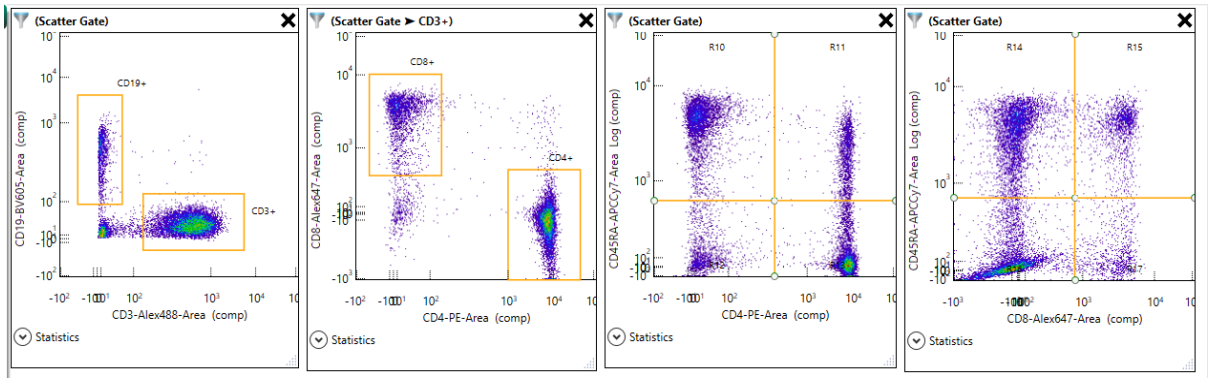
This section applies to the primary ZE5 Cell Analyzer.

To generate the reference assay

1. Create the experiment assay to be standardized on the primary ZE5 Cell Analyzer (reference assay).
2. Based on the needs of your lab, determine the optimal instrument settings (fluorescent channels selected, PMT voltages, compensation values, and so forth) for the reference assay.

   Important: Optimal settings are crucial because the assay will be replicated across time and/or instruments.
3. In Everest Software, run compensation controls and complete the auto-compensation process.
4. Ensure the settings are sent back to the instrument.
5. Collect and record experiment samples.
6. Analyze the results and ensure the data is acceptable. Figure 2 displays an example of assay results.

   Figure 2. Fully stained sample showing population regions of interest



7. Rename the experiment reference assay and add a date and experiment identifier.

Section 2: Measuring Bead Standards

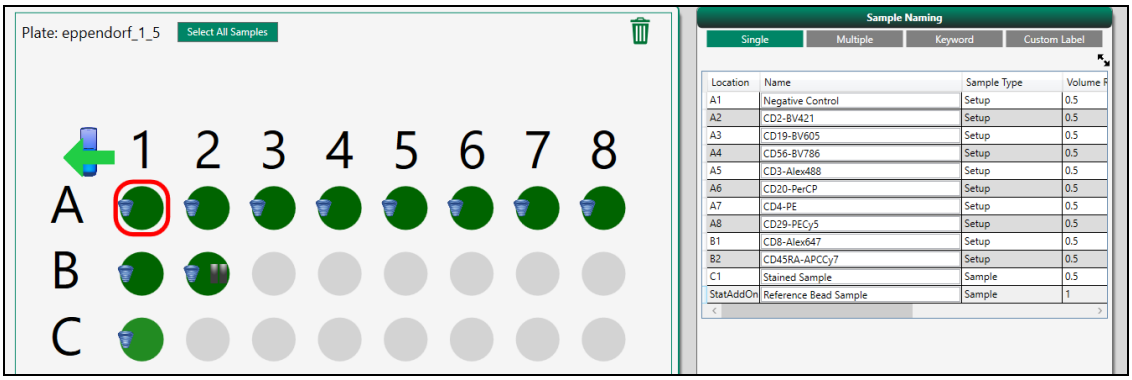
This section applies to the primary ZE5 Cell Analyzer.

To measure the bead standards

1. In Everest Software, select the Plate Setup tab and do the following:

   a) Add a single sample well or stat tube (Figure 3) containing the fluorescent bead standard and enter the Spherotech™ bead lot number as the sample name.

   Figure 3. Plate design with stat tube add-on for Spherotech™ reference beads



   b) Set the Flow Rate to Low.

   c) Set an Event Limit of 10,000 events.

2. Select the Plots and Gates tab and do the following for each fluorescent channel in the assay:

   a) Create FSC/SSC plots.

   b) Use the plot builder to create individual histogram plots (area with the scale set to Log).

3. Prepare a reference bead sample:

   a) Dilute the Spherotech™ beads 1:10 in PBS.

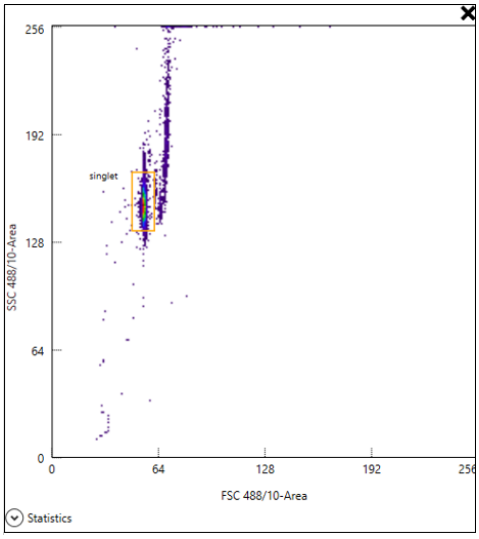
   b) Place the tube in the correct position in the ZE5 tube rack.

4. In Setup mode on the Acquisition screen, acquire the reference bead sample.

5. Examine the scatter in the FSC/SSC plot. If necessary, adjust FSC and SSC PMT voltages to bring the bead population on scale.

6. As shown in Figure 4 on page 6, draw a region around the bead singlet population on the FSC-A/SSC-A plot, and filter to all other plots and histograms for this population.

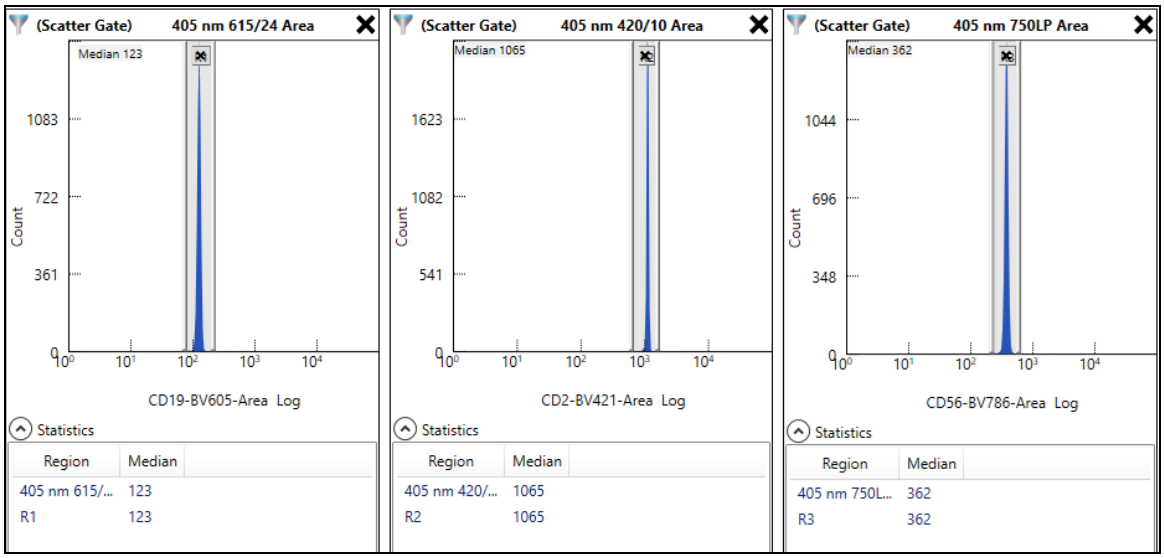
Figure 4. SSC/FSC plot of Spherotech™ beads showing region of interest



7. Change to Acquisition mode and collect 10,000 events of the reference bead sample.
8. Once acquisition is complete:
   a) Continue to the Analysis screen to create a region that tightly encompasses the histogram peak for each histogram (Figure 5).
   b) For each histogram, click the Statistics arrow to display the median fluorescence value statistic on the plot (Figure 5).
   c) Using the plot tools, annotate each histogram with (or note on a separate document) its median fluorescence value. ***These are your Master Fluorescence Values (MFV)***.

   The following graphic displays the histograms of fluorescence intensity (for each fluorescent channel) with the region drawn around the peak. Median values are shown for each peak and annotated on the histogram.

Figure 5 Histograms generated in this section



9. Once complete, click the Send to Local Instrument button to return to the Acquisition screen.

Section 3: Standardizing Future Assay Instances

This section applies to the primary ZE5 Cell Analyzer.

To standardize future assay instances

1. To determine whether the current Observed Fluorescence Values (OFV) are the same as the MFV determined in Section 2, open the reference assay experiment in Edit mode.

   This launches a new session of the experiment with all settings retained.

2. Rename this instance to differentiate it from the original reference assay and open the Acquisition screen.

   Important: Do not modify the experiment.

3. Using the same lot number, prepare a new 1:10 dilution of Spherotech™ beads in PBS and place the reference bead sample into the tube rack.

4. Run the sample in Setup mode and compare the current median OFV in each histogram to the MFV.

   If necessary, adjust PMT voltages to bring the OFV to the same median fluorescence value as the MFV for each channel.

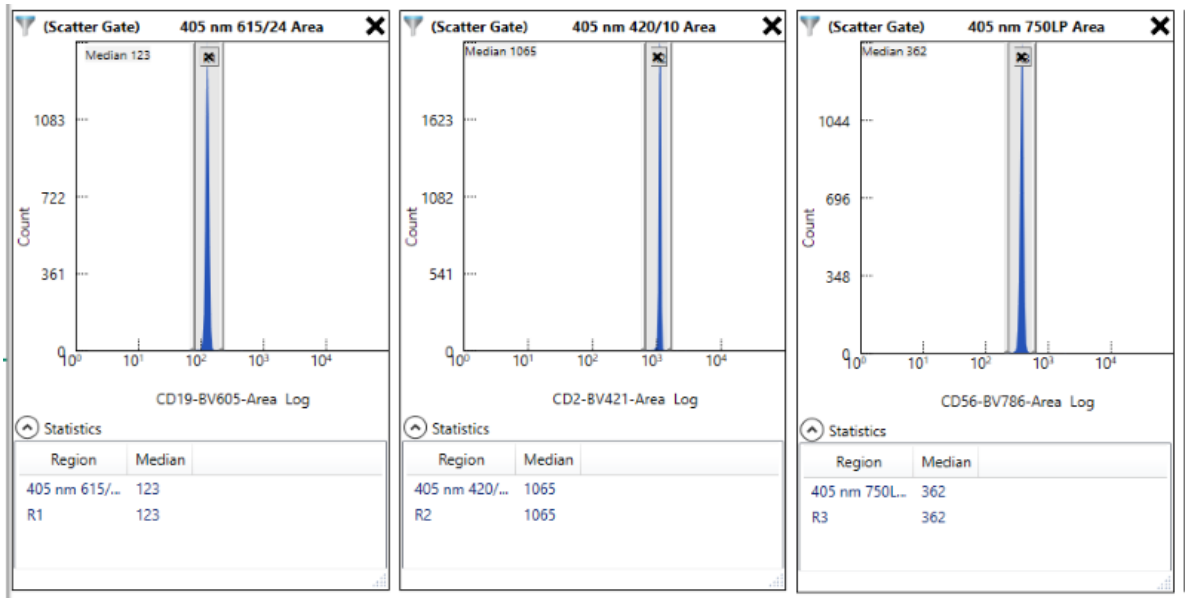
   Note: Identical performance of the reference beads ensures that the primary ZE5 instrument performance has been standardized for this assay.

   5. Once adjustments are complete, you can (optionally) collect a “match to reference” data set to demonstrate that the OFV matches the MFV, as shown in Figure 6. The PMT voltages determined in this example are used to acquire the samples in Step 8.

   The following graphic displays the histograms of fluorescence intensity (for each fluorescent channel) with the region drawn around the peak. Median values are shown for each peak and annotated on the histogram.

   Note: The histograms have identical peaks and median values to those shown in Section 2, Step 8.

Figure 6. Histograms generated in this section



6. Return to the Home screen and edit the updated session of the reference assay, which contains the new PMT voltages.

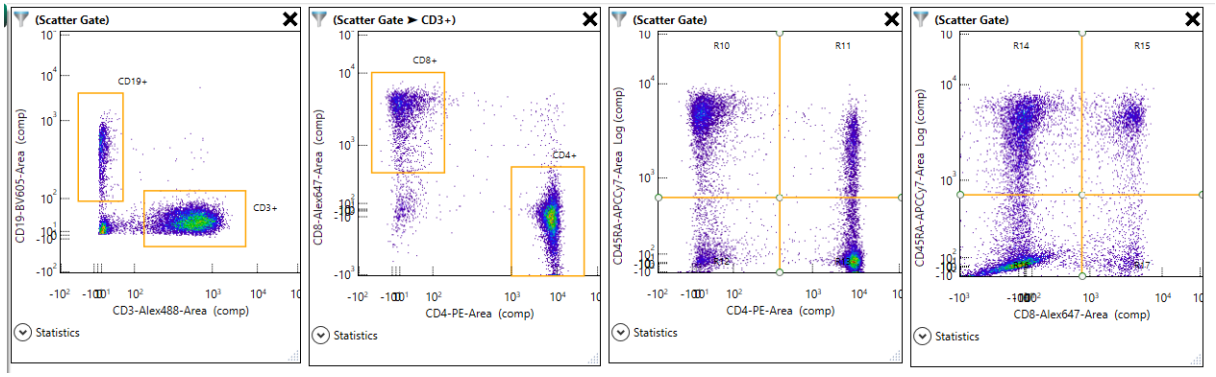
7. Delete the bead sample well and update the samples, as needed, for the current assay run.

   Tip: You can also delete the histograms used to track the reference beads from the Plots and Gates tab workspace.

8. Begin standard sample collection.

   Important: When collecting experiment samples, you might need to adjust SSC and FSC PMT voltages to bring the sample population into view in the SSC/FSC plot. **Do not modify PMT voltage values for any fluorescence channels or standardization is lost.** When finished, you can record the beads again as a final post tube and use the same settings to confirm the primary instrument did not experience any drift during the run. An example of experiment data captured at this step is shown in Figure 7.

Figure 7. Results of experiment assay using standardized settings



Note: Substantial differences in the median fluorescence values can indicate a significant change has occurred in the primary ZE5. Check your QC Trending Report for such a change in the performance of the affected channels.

Inter-Instrument Protocol

In this protocol, the reference assay created on the primary ZE5 in the Inter-Assay protocol is transferred to the secondary ZE5 to standardize the same experiment assay across instruments.

Note: Once determined, keep the primary and secondary instrument designations consistent throughout execution of this protocol.

Section 1: Transferring the reference assay from the primary instrument to the secondary instrument.

Section 2: Running the same lot of Spherotech™ bead samples to calibrate the ROFV (median values) on the secondary instrument.

Section 1: Transferring the Reference Assay

This section applies to the secondary ZE5 Cell Analyzer.

To transfer the reference assay

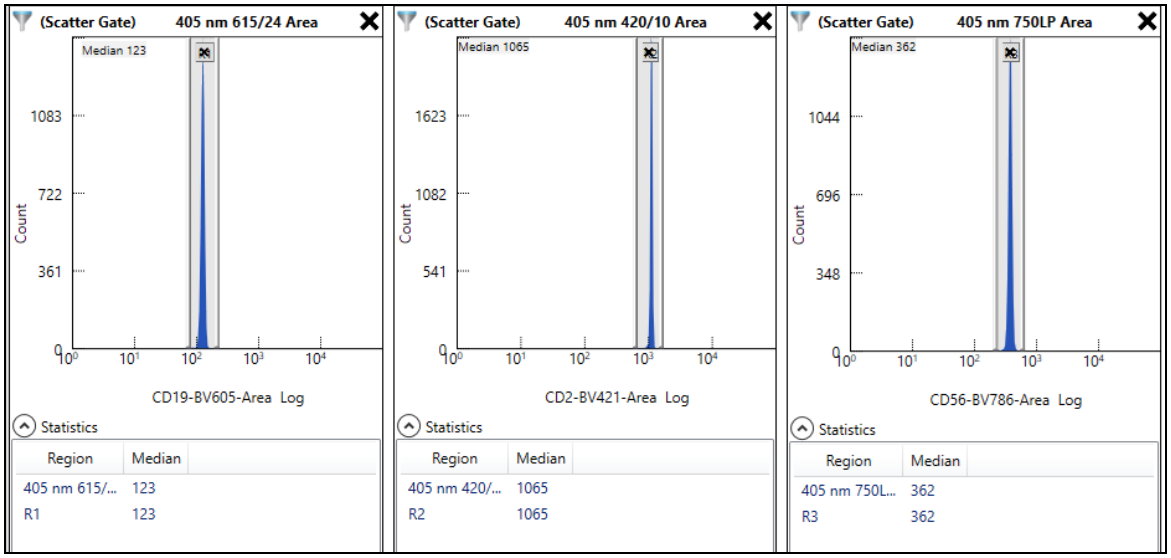
1. Save the reference assay experiment created in the Inter-Assay Protocol, including the data, to an external hard drive or a secure online storage location.
2. Open Everest Software for the secondary instrument and select the Analysis tab, and then click Load Experiment.
3. Navigate to the reference assay location, and select the reference assay.
4. Verify the results are the same as in the Inter-Assay protocol. As shown in Figure 8, click Send to Local Instrument to transfer all PMT voltages and plots to the Acquisition dashboard.

Figure 8. Dashboard showing the Send to Local Instrument button

5. Create a freshly prepared 1:10 dilution of the same lot number of Spherotech™ beads in PBS and load the reference bead sample into the tube rack.
6. Run the sample in Setup mode and compare the current median ROFV in each histogram to the MFV. If necessary, adjust PMT voltages to bring the ROFV to the same median fluorescence value as the MFV for each channel.

The following graphic displays the histograms of fluorescence intensity. Median values are shown for each peak and annotated on the histogram. These median values should match the median values generated on the primary instrument.

Figure 9. Histograms generated on the secondary instrument



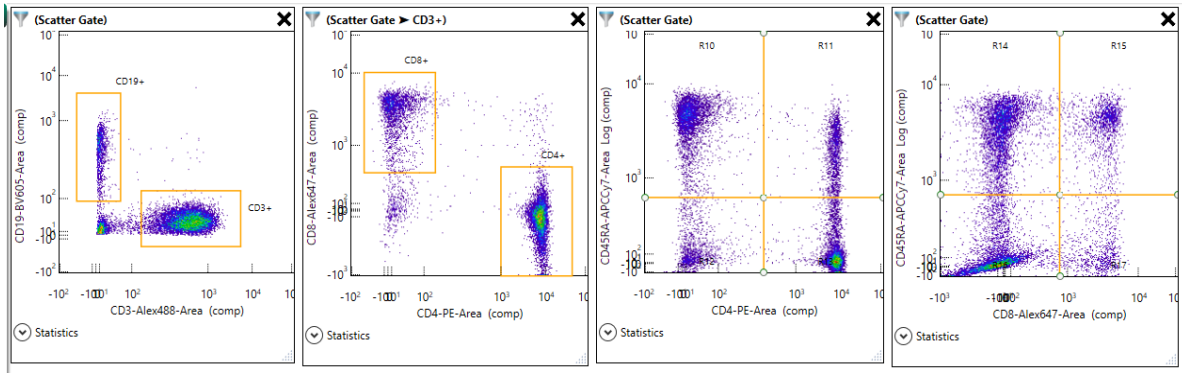
Note: Identical performance of the reference beads ensures the *primary* instrument performance has been standardized for this assay.

7. Return to the Home screen and edit the updated session of the reference assay, which contains the new standardized PMT voltages.
8. Delete the bead sample well and update the samples, as needed, for the current assay run. You can also delete the histograms used to track the reference beads from the Plots and Gates workspace.
9. Begin standard sample collection.

   Important: When collecting experiment samples, you might need to adjust SSC and FSC PMT voltages to bring the sample population into view in the SSC/FSC plot. **Do not modify PMT voltage values for any fluorescence channels or standardization is lost.** When finished, you can record the beads again as a final post tube and use the same settings to confirm that the secondary ZE5 instrument did not experience any drift during the run. An example of experiment data captured at this step is shown in Figure 10 on Page 11.

The following graphic shows the experiment results on the secondary instrument, after adjusting PMT voltages to calibration fluorescence measurements using the fluorescent bead standard.

Figure 10. Experiment results on the secondary instrument



Note: You might need to re-run compensation control wells; however, the primary and secondary instruments should generate similar compensation matrices. It is the user's responsibility to validate the method in line with laboratory standards.

Section 2: Updating the Reference Bead Values

Reference values might need to be reset on occasion; for example, when changing to a new lot of fluorescent bead standards, or after significant instrument maintenance. You can achieve the best results if you perform this protocol before the current bead lot is depleted.

This section applies to the primary ZE5 Cell Analyzer. The new values are used as a reference for setting the secondary instruments.

To update the reference bead values

1. Run the reference assay using the current fluorescent bead standard lot (Lot 1).
2. Modify PMT voltages for each fluorescence channel to reproduce the reference assay MFV.
3. Run the new fluorescent bead standard lot (Lot 2) using the PMT voltage values determined in Step 2 and record the values produced.

Going forward, these MFV serve as the new reference assay MFV.

Contacting Technical Support

The Bio-Rad Technical Support department in the U.S. is open Monday through Friday, 5:00 AM to 5:00 PM, Pacific Time.

Phone: 1-800-424-6723, option 2
Email: Support@bio-rad.com (U.S./Canada Only)

For technical assistance outside the U.S. and Canada, contact your local technical support office or click the Contact Us link.

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