
Rainbow Bead Standardization – Attune

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Why Standardize My Flow Assay?

Assay standardization in flow cytometry improves reproducibility and reduces cytometer set-up time. For assays evaluating protein expression levels using MFI (median fluorescence intensity), this standardization is critical for reducing user set-up variation among replicates. For all flow assays, standardization expedites cytometer set-up and data analysis.

How Does Rainbow Bead Standardization Work?

The observed fluorescence intensity on a flow cytometer is dependent on many non-biological parameters including laser power, alignment, temperature, and optical efficiency. When setting up an experiment, we adjust the sensitivity of the detectors to optimize instrument performance for the run, but these voltages may not be optimal for the next week or even the next day and adjustments must be made each time the assay is run. Rainbow beads (Spherotech, Cat. RFP-30-5A Rainbow Fluorescent Particles Mid-Range) are hard-dyed fluorescent particles that emit as a single peak in each instrument channel. The core can provide these beads at our reduced cost. Acquiring these beads under optimized instrument settings generates a set of target values that reflect instrument sensitivity. For future runs, acquire first and adjust voltages until the bead peaks hit the target values (as close as possible). At this point, set-up is complete and collection of compensation and test samples can proceed. This method of standardization is very robust and able to accommodate both daily variations and larger changes in performance such as would be seen after cytometer maintenance and alignment.

Do I Still Have to Compensate?

Yes! Standardization tells you where to set voltages (instrument sensitivity) using multicolor beads, but compensation uses single stained controls to account for fluorescence spillover. Compensation is still critical for obtaining good multicolor flow cytometry data. For the most accurate compensation, there are three basic rules that must be followed:

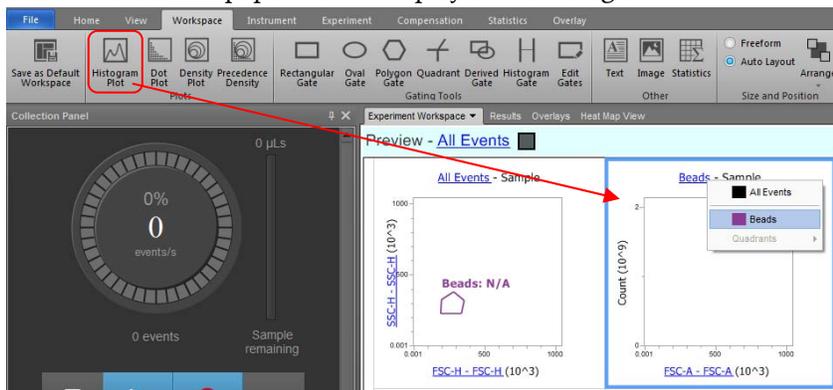
1. The compensation control must **be as least as bright** as the stained sample.
2. The negative and positive populations must have **matched autofluorescence**: cells to cells, beads to beads, etc.
3. The fluorochrome used for the control must **exactly match the fluorochrome** used in the experiment. FITC cannot be used instead of GFP, tandem dyes must be from the same vial as the experimental stain.

How Do I Standardize on the Attune?

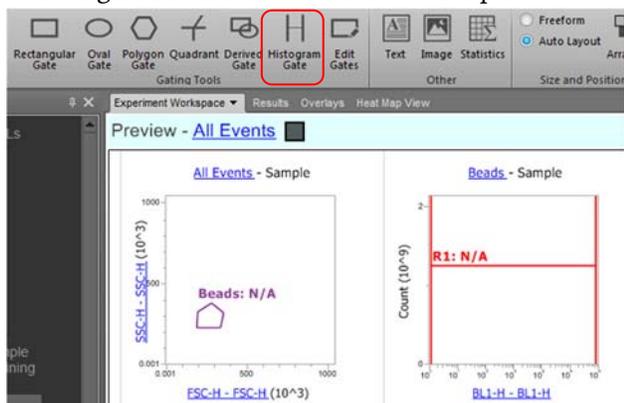
1. Set optimized voltages manually using a fully stained sample and checking any controls you wish before finalizing voltages (do not record, calculate, or apply compensation yet).
2. Optionally, add empty text boxes to fill empty spaces on the current page of the workspace (so that all bead plots land on page 2 – this is purely cosmetic).
3. Make an FSC-H/SSC-H dot plot displaying All Events, and draw a region called Beads.



4. Make a histogram plot for the first fluorescence channel (e.g. BL1-H).
5. Select Beads as the population to display on the histogram.



6. Draw a gate across the entire width of the plot.



Preparing Rainbow Beads

Dilute one drop of well-mixed rainbow beads in 300 μ L of flow buffer.

Rainbow Initial Set-up Quick Reference

1. Optimize cytometer voltages using your samples.
2. Collect the rainbow beads without any compensation and record the median fluorescence intensity for each channel.
3. Collect and apply compensation.
4. Acquire your samples.

Set-up Already Standardized Assay

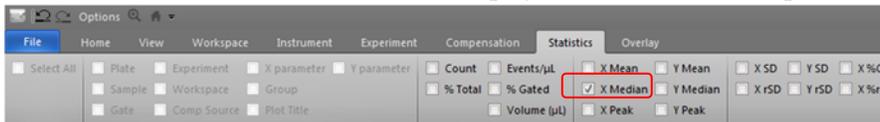
1. Acquire rainbow beads and adjust detector voltages to hit MFI target values determined as above.
2. Proceed with compensation.
3. Acquire samples.

How Do I Change Bead Lots?

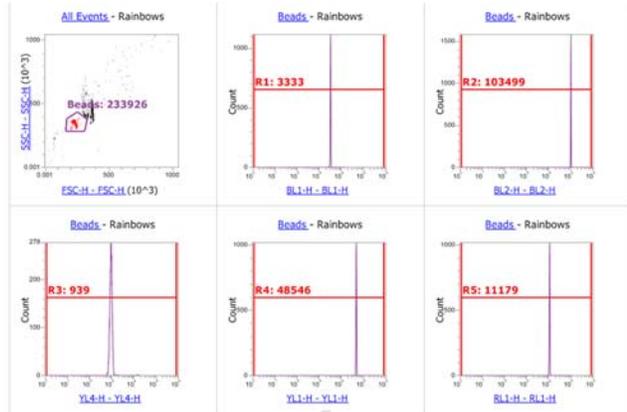
Changing bead lots is simple as long as you have beads from both lots.

1. Run old bead lot and adjust voltages to hit target values.
2. Acquire new bead lot at those settings.
3. Note the MFI in each channel for the new bead lot – these are your new target values.
4. Update the text box with the new lot number and MFI target values to use in future experiments.

- In the Statistics ribbon, select the box to display the X Median on the plot.

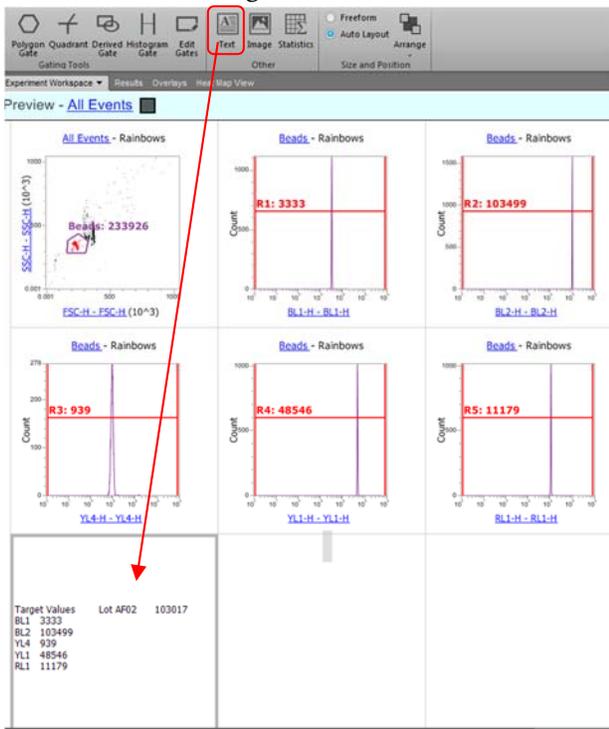


- Copy and paste the histogram plot to make one plot for each channel in the experiment, and change the X axes so each channel is represented once in the worksheet.



- Record a data file of 10,000 events from the Beads gate, adjusting the gate if needed. The median values from the histogram plots are your target values.

- Create a Text Box at the bottom of the worksheet and type the name and target value for each channel. This way, if you save a template your target values will be listed right on the worksheet.



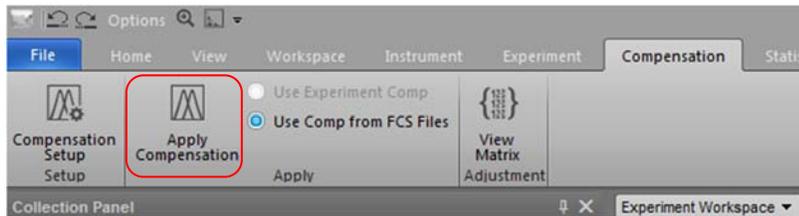
- Proceed with compensation and sample acquisition.

- Save a Template to use in the future.

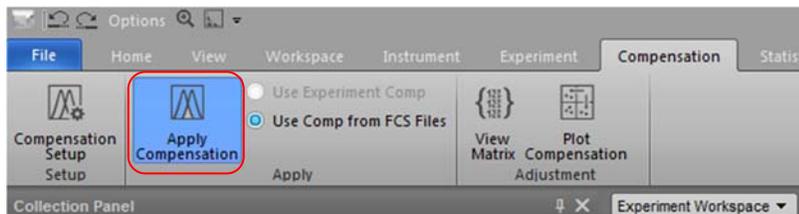
Running Your Assay After Standardization

1. Load the appropriate template.
2. Scroll to the page of Rainbows plots.
3. Acquire beads and adjust voltages until the median values displayed on the histograms match the target values in the text box (as close as possible).

Important note: **Make sure the software is not applying compensation to the tube!** Compensation will affect target values. Check for compensation in the Compensation ribbon at the top of the window. Make sure the “Apply Compensation” button is not highlighted in blue, and click to remove compensation if it is currently being applied.



When the Apply Compensation button is grey, compensation not applied This is what you want when looking at Rainbow beads.



When the Apply Compensation button is blue, compensation is applied. Click the button to turn it grey and remove compensation before looking at Rainbow beads.

4. Proceed with compensation and sample acquisition.