WG2 SOP-01 Flow Cytometer Setup and Performance Characterization

1. Subtitle

Verification of Flow cytometer Performance Using Rainbow 8 Peak Beads and FC beads

2. Purpose and Introduction

The Spherotech 8 Peak Rainbow Calibration Particles (RCPs) contain a mixture of particles with different fluorescence intensities. One particle population is unstained and 7 populations contain a mixture of fluorophores that allows excitation at any wavelength from 365 to 650 nm. As a result, the Rainbow 8 Peak Beads can be used for the evaluation of most channels in the flow cytometer, including the calibration of the intensity scale in unites of statistical photoelectrons (Spe). Eight different preparations of BD Biosciences FC beads, each labeled with one specific fluorophore and assigned with an ERF (equivalent reference fluorophore) value, are used to calibrate the fluorescence intensity scales in units of ERFs. This SOP is used to prepare for the calibration of the fluorescence response, in units of Spe and ERF, and to estimate the background (B, in units of Spe and ERF) and the detection efficiency (Q, units of Spe/ERF), which can be used to estimate the limit of detection and to predict instrument performance in resolving particles of different brightness.

3. Reagents and Materials

Name/Material Code	Vendor	Catalog# Lot/Batch #	Storage Conditions	Reconstitution	Supplied by
Rainbow 8 Peak Calibration Particles RCP-30-5A	Spherotech	RCP-30-5A	2-8°C, protected from light	See below	NIST
FC-FITC	BD Biosciences	661615 2046553	2-8°C, protected from light	See below	NIST
FC-PerCP-Cy5.5	BD Biosciences	661619 2101886	2-8°C, protected from light	See below	NIST
FC-PE	BD Biosciences	661616 2061631	2-8°C, protected from light	See below	NIST
FC-PE-Cy7	BD Biosciences	661617 2073743	2-8°C, protected from light	See below	NIST
FC-APC	BD Biosciences	661620 2073756	2-8°C, protected from light	See below	NIST
FC-APC-Cy7	BD Biosciences	661622 2070363	2-8°C, protected from light	See below	NIST
FC-V450	BD Biosciences	661623 2115671	2-8°C, protected from light	See below	NIST
FC-V500-C	BD Biosciences	661624 1348847	2-8°C, protected from light	See below	NIST
FC Beads Reconstitution Buffer	BD Biosciences		2-8°C, protected from light	See below	NIST
Milli Q Water					

3.1. Supplies

- Staining Tubes: 5 mL Polystyrene Round-Bottom Tubes (BD Falcon Cat. No. 352008 or equivalent) or Microwell Plates (Sarstedt Cat No 82.1583, Corning Cat No 351172, or equivalent)
- Pipet tips [P1000]
- Sterile serological pipette (1mL)

3.2. Equipment

- Pipettor P1000
- Pipette-aid
- Flow Cytometer

3.3. Reagent Preparation

Rainbow 8 Peak Calibration Particles

1. Add 2-3 drops of Rainbow 8 Peak Calibration Particles to 1 mL of Milli Q Water.

FC Beads

2. Add 0.5 mL of FC bead reconstitution buffer to a FC bead tube and vortex gently for 20 second.

4. Procedure

4.1. Instrument Setup

1. Perform the daily instrument QC & calibration per your institution's SOP (Note: cytometer fluorescence detector gain/voltage and laser power settings are the same as those used for immunophenotyping per laboratory SOP).

Turn off compensation if it is not already off after daily QC.

Note for spectral cytometers: *data will be acquired as raw and not an unmixed data file.*

#1 Recommendation: The detector gain settings used for immunophenotyping are the preferred settings (baseline settings), however the three conditions also have to be met: 1) at least 5 fluorescent Rainbow bead populations of the 8-peak beads are distinguishable for all 8 fluorescence channels; 2) all 8 fluorescent FC beads are on scale in their respective fluorescence channel; 3) a set of bead compensation controls, either AbC total antibody compensation kit plus pacific blue beads and Aqua beads, or Versacomp antibody capture bead kit plus pacific blue beads and Aqua beads (see SOP-2 for their preparations), are on scale. Final detector gain settings require that the fluorescent FC bead population and compensation beads are all on scale with 5 distinguishable Rainbow bead populations shown in each fluorescence channel. If necessary, reduce detector gains to ensure all three conditions are met.

#2 Recommendation: If there is not a setting used for routine immunofluorescence analysis for human blood phenotyping, please take the following steps: 1) adjust the detector gains so that

the brightest population of the 8-peak Rainbow beads is on scale; 2) decrease or increase the detector's gain settings with FC beads to ensure all FC beads are on scale; 3) double check the detector gains with a set of bead compensation controls, either AbC total antibody compensation kit plus pacific blue beads and Aqua beads, or Versacomp antibody capture bead kit plus pacific blue beads and Aqua beads (see SOP-2 for their preparations), are on scale. Again, the final detector gain settings require that the fluorescent FC bead population and compensation beads are all on scale with 5 distinguishable Rainbow bead populations shown in each fluorescence channel.

Example plots of 8-peak Rainbow beads, FC beads, and Versacomp antibody capture beads plus pacific blue- and Aqua-coated beads are shown in Figure 1, Figure 2, and Figure 3 in Appendix A, respectively.

Notes:

1) A set of bead compensation controls should be evaluated to optimally set up gain settings for the entire study. The fluorescence intensities of all 8 bead compensation controls are higher than the respective cell-based compensation controls. In particular, the pacific blue- and Aqua-coated beads are highly fluorescent. Though it is not mandated to acquire the set of bead compensation controls, the bead compensation controls stored in dark in 0-4 °C refrigerator are stable and can be used on the second day and monitor the consistency of the compensation matrix determined (also see Note #2 below).

2) The 8-peak Rainbow beads serve as the monitoring beads for the stability of day-to-day instrument stability under the optimized gain settings. An acceptable range of %CV is dependent of fluorescence channel, e.g., 1.4% for FITC channel and 3.4% for PE-Cy7 channel and should be established. Participant can choose one of middle fluorescence intensity populations of the 8-peak beads as the monitoring bead population to define your instrument tolerance range of the variability in Day 1 and day 2, e.g., CV of 2% for FITC channel, when the same compensation bead controls, e.g., Versacomp beads plus PB- and Aqua-coated beads, are run and the compensation matrixes are obtained and compared. Only under the acceptable range of variability, no new compensation controls need to be run on different experimental days.

3) FC beads after reconstitution with a 0.5 mL of the reconstitution buffer can be run for a few tests and are stable for at least two days in dark in 0-4 °C refrigerator.

Note for spectral cytometers: *Altering individual detector settings with spectral cytometers is not recommended. Instead, alteration of all detector channels should be made through "All Channels %" found in instrument control of Aurora cytometers. For spectral cytometers it's highly advised to fully characterize your instrument across the commonly used parameter channels in order to establish routine detector settings for immunofluorescence analysis for immunophenotyping. [A bioassay manuscript on the settings for immunofluorescence analysis could serve as a reference (https://pubmed.ncbi.nlm.nih.gov/34708658)]*

2. Create a WG2 Rainbow 8 Peak Acquisition Template (Figure 1).

3. Acquire the bead in setup mode at a relatively slow flow rate.

4. Note that beads are smaller than blood cells, therefore the FSC and SSC gains will need to be increased in order to place the beads on scale in the FSC vs SSC plot. Set a gate for the singlet beads population on the FSC vs SSC bivariate plot to exclude background particles/noise, and bead doublets and higher order aggregates (Figure 1).

5. Note that the Relative Channel Number of the initial dot display screen may look cluttered due to the presence of background, bead doublets and higher order aggregates. However, after setting a gate on singlet beads using their FSC vs SSC intensities, the dot display screen as well as FSC histogram (Figure 1) reflects the intensities of single beads.

   Gating on the bead population in the FSC histogram, it's desirable to resolve as many fluorescent bead populations as possible for each of the 8 fluorescence channels shown in the fluorescent histograms (Figure 1, Use pulse area, not pulse height) under the PMT/gain setting for either Recommendation #1 or #2 depending on participants' situation. Some flow cytometers may not be able to resolve all 8 intensity populations in every detector. However, there should be detectors, e.g., detectors for FITC, APC, and APC-Cy7 where all or most of the populations can be resolved (Figure 1). A minimum of 5 intensity populations is required for sensitivity determination; the mean or median, robust SD, and robust CV of these resolvable populations can be determined. Also see Figure 1 for example of Y763-PC7 (PE-Cy7) detector that does not resolve the dimmest populations but has shown 5 distinct populations for statistical analysis on cytometer performance.

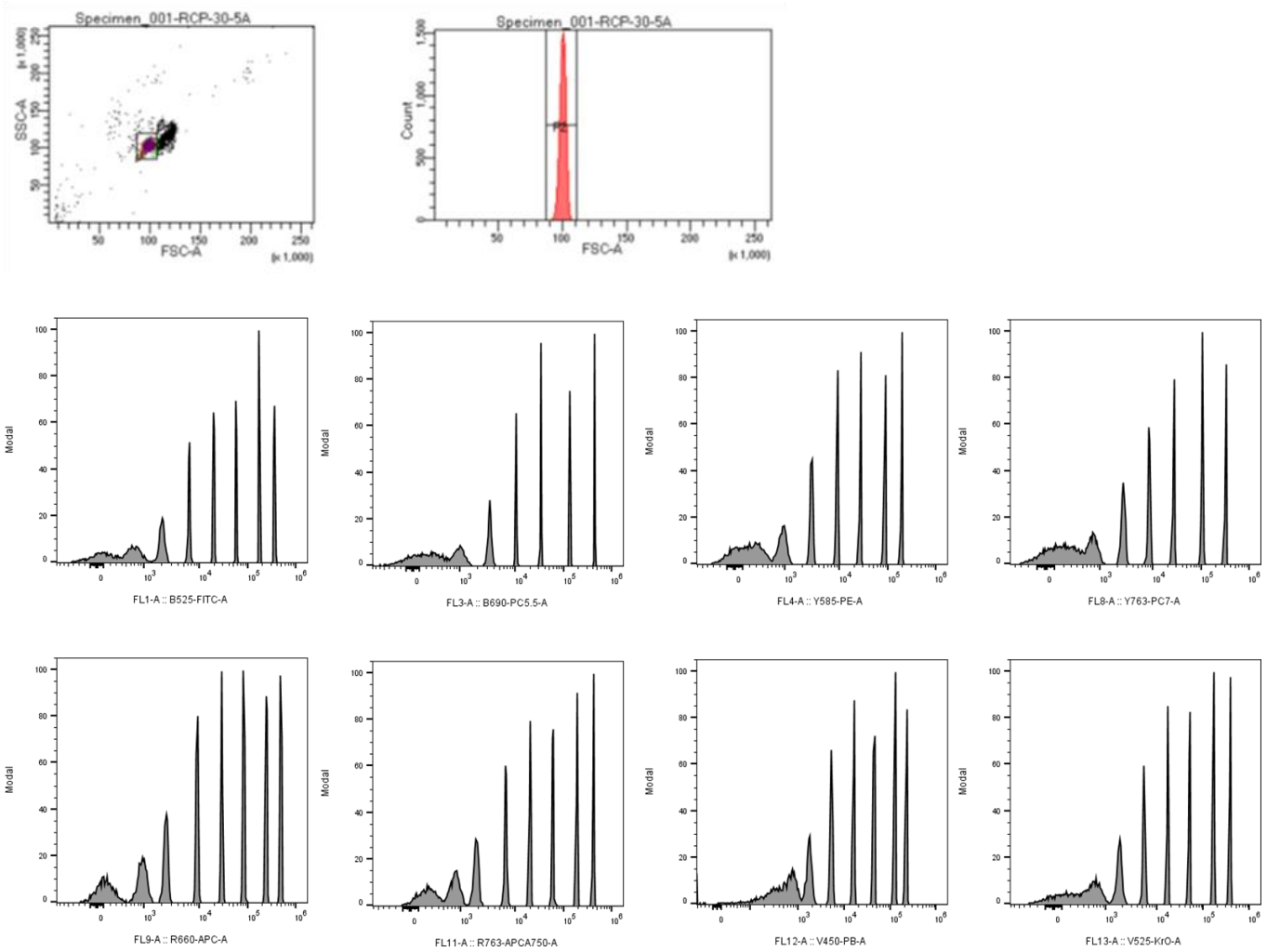

Figure 1. Example of Rainbow 8 Peak Bead Acquisition made by using a CytoFLEX LX. In this example the brightest fluorescence intensity population is on scale for all 8 fluorescence channels, however, not all the populations are resolved. The protocol requires that at least 5 intensity populations are distinguishable for the analysis of flow cytometer performance characteristics. For CytoFLEX LX: B525-FITC and B690-PC5.5 (PerCP-Cy5.5) are under 488 nm laser excitation; Y585-PE and Y763-PC7 (PE-Cy7) are with 561 nm laser excitation; R660-APC and R763-APCA750 (APC-Cy7) are under 638 nm excitation; V450-PB and V525-KrO (Aqua) are with 405 nm excitation.

6. Repeat the Step #4 above on 8 FC beads to ensure FSC and SSC gains are properly set as shown in Figure 2. The FSC and SSC gains can be adjusted if needed. Verify all 8 FC beads on the 8 different fluorescence channels are on scale in their respective histograms, and if necessary, reduce the detector voltage so the FC population is on scale. **It is essential that the FC beads and Rainbow beads are acquired under the same fluorescence gain settings for the 8 fluorescence channels.** Example histograms for FC beads are provided in Figure 2.

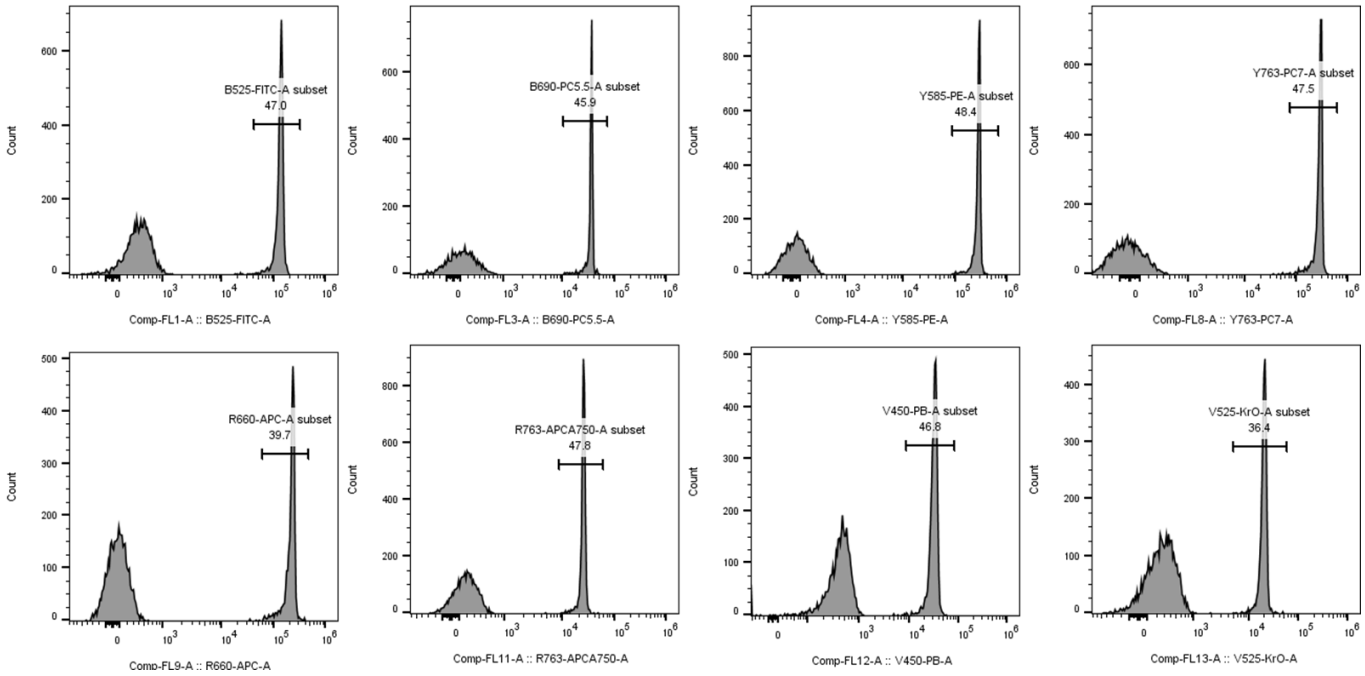

Figure 2. Example of FC Bead Acquisition made by using CytoFLEX LX. For CytoFLEX LX: B525-FITC and B690-PC5.5 (PerCP-Cy5.5) are under 488 nm laser excitation; Y585-PE and Y763-PC7 (PE-Cy7) are with 561 nm laser excitation; R660-APC and R763-APCA750 (APC-Cy7) are under 638 nm excitation; V450-PB and V525-KrO (Aqua) are with 405 nm excitation.

7. Repeat the Step #6 above on a set of bead compensation controls, e.g., Versacomp beads plus PB- and Aqua-coated beads to ensure all positive bead populations in respective fluorescence channels are on scale as shown in Figure 3 of Appendix A. Only FSC and SSC gains can be adjusted and if necessary, reduce the detector gain so the positive bead population is on scale.

8. Use the scatter gates for Rainbow Beads and the final detector gain settings to create an acquisition sample tube/well for Rainbow beads.

9. Use the scatter gates for FC beads and the final detector gain settings to create 8 acquisition sample tubes/wells for FC beads. Assure that the detector gain settings are the same for both the Rainbow and FC bead acquisition templates.

4.2. Acquisition (Exp ID-e1)

1. Open the WG2 Rainbow 8 Peak Acquisition Template (or Experiment workflow) and 9 sample tubes with local file names for Rainbow 8-peak beads and 8 different FC beads.

2. Acquire a minimum of 20,000 events in the singlet/major population gate, e.g., P2 gate in Figure 1 and 8 fluorescent FC bead gates in Figure 2.

4.3. Local Filename Entry on the WG2 Instrument Reporting Spreadsheet

1. Open the WG2 Reporting Workbook and select the "Instrument" Tab. Verify that the Study ID, Site ID, Instrument ID and Protocol ID at the top of the Spreadsheet describe the current execution of the protocol. See Figure 4 for example. One Workbook is required for each instrument.

2. For each FCS file collected (9 total files) find the corresponding material code row using the terms in "Column C". See Figure 4.

3. Identify the material associated "Get Local Filename" button in Column O to identify the local FCS file location. See Figure 4.

4. Press the button and use the file dialog box to identify the local FCS filename corresponding to the files collected in section 4.2.

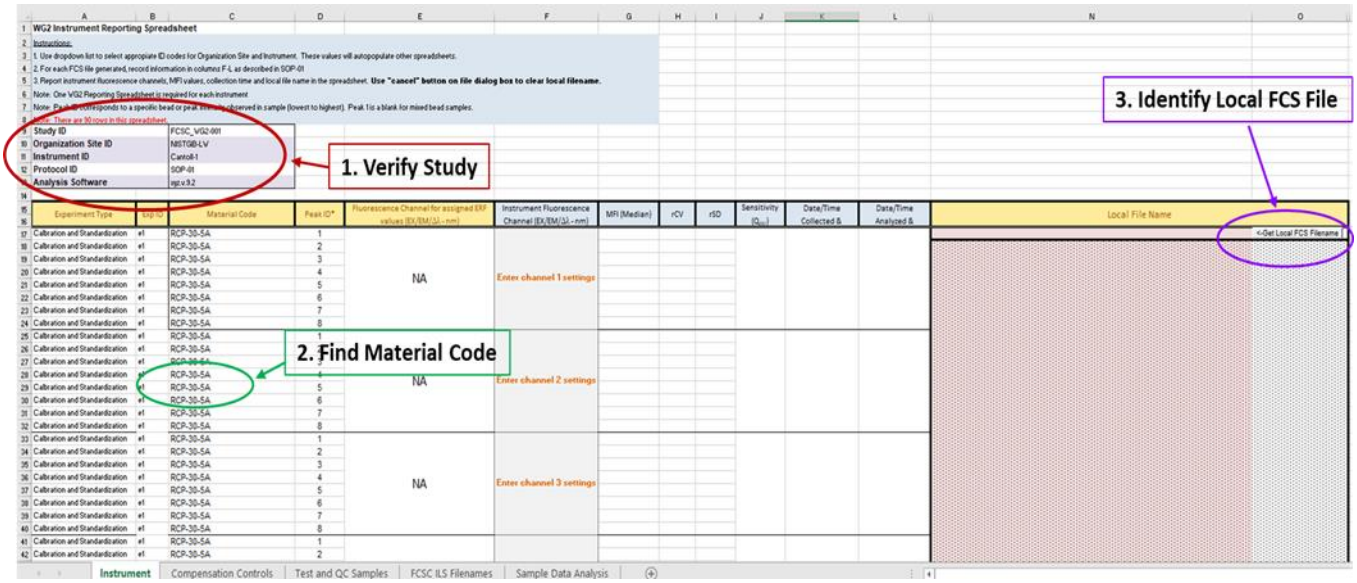

Figure 4. WG2 Instrument Reporting Spreadsheet.

4.4. Data Analysis and Reporting for Instrument Setup and Performance Characterization

1. Import the .fcs files of Rainbow 8-peak beads and 8 FC beads from the acquisition to a data analysis software.

2. Use the gating strategies shown in Figure 1 and 2 and create a statistical report table to obtain Median Fluorescence Intensity (MdFI), robust standard deviation (rSD), and robust coefficient of variation (rCV) for all measurable Rainbow bead peaks and fluorescent FC bead peak. Note: for Rainbow 8-peak beads, peak 8 is defined as the most fluorescent bead population and peak 1 is for the blank bead population.

3. Record MdFI values and associated rCV and rSD for bead populations/peaks in the Column ‘G’, Column ‘H’, and Column ‘I’, respectively, in the ‘Instrument’ sheet of the WG2 Reporting Spreadsheet.

4. Fill in information on the instrument fluorescence channels, dates for data acquisition and analysis, and operator(s) in Columns ‘F’, ‘K’, and ‘L’, respectively in the ‘Instrument’ sheet.

5. Attachments

1. Spherotech Rainbow 8 Peak Calibration Particles
2. BD FC Bead Information

Appendix A: Plots of a set of bead compensation controls under the same detector gain setting for 8-peak Rainbow beads (Figure 1) and FC beads (Figure 2)

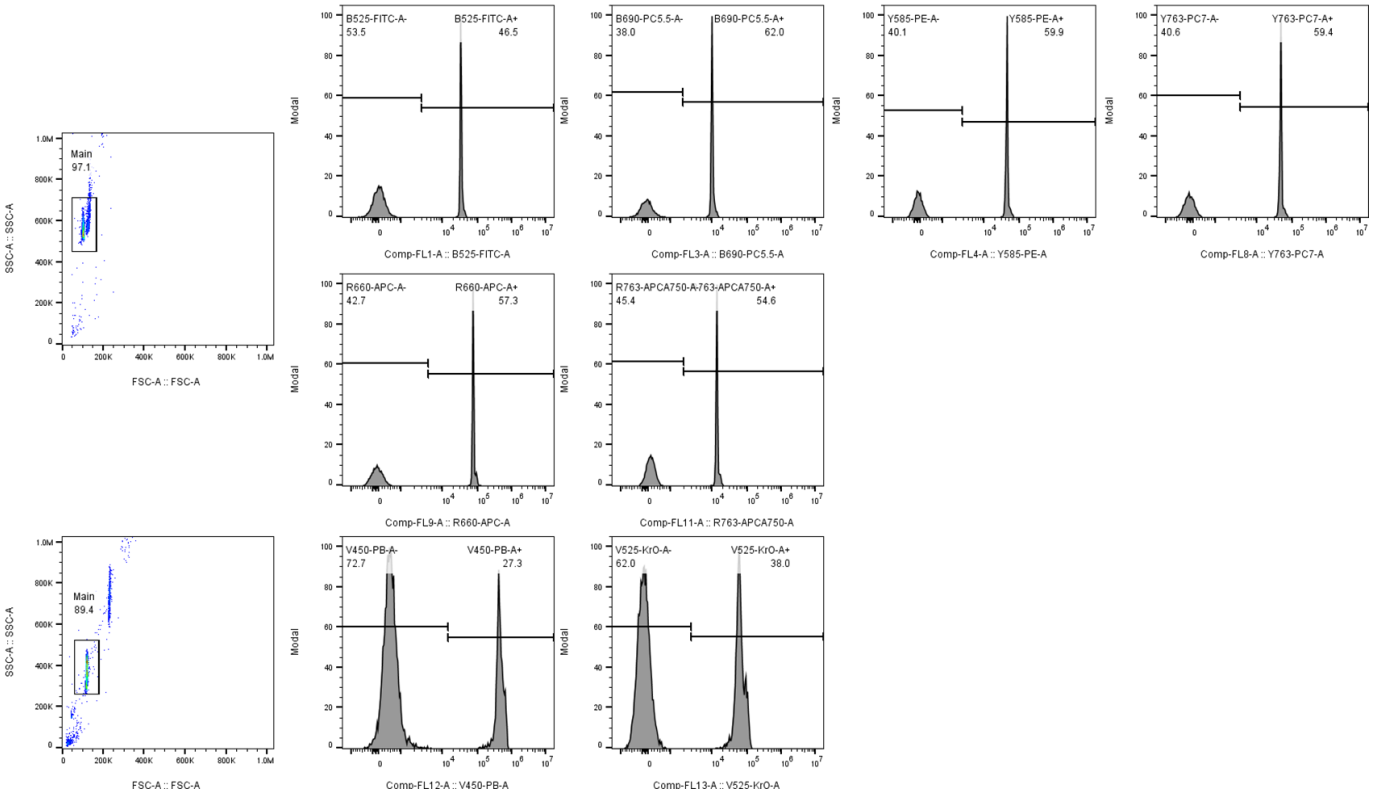

Figure 3. Example plots of Versacomp antibody capture beads (7 plots on the upper rows) plus pacific blue- and Aqua-coated beads (3 plots on the bottom row) acquired by using CytoFLEX LX. **Note**: the sizes of Versacomp antibody capture beads and Pacific blue-/Aqua- beads are different, and hence the gains for FSC and SSC need to be adjusted for proper detection (See the respective plots of FSC-A vs. SSC-A).