

Designing BD™ CBA Flex Set Templates for Flow Cytometers Running BD FACSDiva™ Software

The following setup procedure is for the BD FACSCanto™, BD™ LSR II, and BD FACSAria™ flow cytometers.

1. Prepare 5 tubes labeled: A9, PE-F1, F1, F9, and A1. Vortex the stock vials of beads, add 200 µl of Wash Buffer to each tube followed by 25 µl of the corresponding setup beads.
2. Edit the parameters list to display only the following: FSC-A, FSC-W, SSC-A, SSC-W, PE-A, APC-A, and APC-Cy7-A.
3. On a global worksheet, create the following plots: FSC-A/SSC-A dot plot, APC/APC-Cy7 dot plot, and PE histogram.
4. Set FSC-A and SSC-A to Log and create a statistics view showing the FSC-A and SSC-A means. Set the events to display to 500. Using the A9 setup beads, adjust FSC and SSC so that the singlet beads have a mean of 30,000 for each parameter. Stop acquisition to avoid running out of sample.
5. Adjust the FSC-A and SSC-A thresholds using the mean channel as a guideline. Be sure that the thresholds do not cut into the bead population.
6. In the FSC-A vs SSC-A dot plot, create a region that includes the singlet population of beads. In the Population Hierarchy, rename that region *singlet*.
7. Edit the statistics view to display the PE, APC, and APC-Cy7 mean of the singlet beads.
8. Through the singlet gate, run the A9 setup beads and adjust the APC and APC-Cy7 voltages until the mean of each parameter is $160,000 \pm 2,000$.
9. Through the singlet gate, run the PE-F1 tube and adjust the PE voltage so that the mean is 65 ± 5 .
10. Create compensation controls and delete the PE compensation tube. Run beads as follows for compensation controls:
 - Unstained: F1
 - APC Stained: F9
 - APC-Cy7 Stained: A1
11. Calculate compensation.
12. Optional: Verify instrument settings prior to analyzing the assay by recording a sample using the remaining mixed capture beads from the Flex Set assay, export as FCS2.0, and go to Tools > Clustering Test in FCAP Array to see if it can identify the correct number of bead clusters.

Set events to record to 300 events per analyte (eg, $300 \times 6 = 1800$ events for a 6 plex) and set the singlet gate as the storage gate and stopping gate to ensure that only singlet bead events are recorded. Change events to display to 5,000. Record samples and export as FCS2.0 files for analysis in FCAP Array.

The Experiment can be saved as a template for future experiments, however it is recommended to verify instrument settings (ie, voltages and compensation) prior to each experiment.



Troubleshooting

Problem	Possible Solution
Bead clusters merging or failure to identify correct number of clusters	Check daily QC results for indications of poor laser alignment (increased CV) or PMT sensitivity (decreased MFI at QC instrument settings).
	Verify that the Window Extension is appropriately set. Approximate values are as follows: BD LSR II: 10 BD FACSCanto: 7 BD FACSAria: 2
	Adjust flow rate. Resolution can improve at lower flow rates.
Debris (FSC/SSC) during sample acquisition	Increase the threshold or enable dual FSC and SSC thresholds. Establish the bead singlet gate as the storage gate and the stopping gate. Use caution as excluded events cannot be recovered post-acquisition.
Low event count	The beads can precipitate, thoroughly vortex individual capture bead bulk vials prior to preparation of master bead mix and vortex the master bead mix prior to dispensing into the individual sample tubes. Vortex sample tubes prior to acquisition.
	Ensure the stopping gate, storage gate, singlet gate, and thresholds are set correctly.
	Avoid aspiration of beads during wash step.

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