BD FACSCalibur™ Flow Cytometer

BD™ CBA Flex Sets: Instrument Setup, Data Acquisition, and Analysis

Instruction Manual

BD

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Introduction

The BD FACSCalibur™ flow cytometer can be used to acquire the BD™ Cytometric Bead Array (CBA) Flex Set assay. This manual provides the information necessary to properly set up the instrument for this assay.

The BD FACSCalibur can accurately distinguish the dual-color BD CBA Flex Set beads in the 30-plex comprised of the beads between A4 – E9 (Figure 1). However, due to the inherent properties of the dyes used in the beads, the BD CBA assay reporter, phycoerythrin (PE), and the instrument optics, the actual distinguishable plex size may be less than 30. This lower number of distinguishable bead populations is because one of the dyes used to index the beads and PE are both excited by the blue (488 nm) laser. Thus, compensation between the bead channel (FL3) and the reporter channel (FL2) must be established. In instances where the PE signal is high (eg, high analyte concentrations) the PE signal spills over into the FL3 channel, causing an increase in mean fluorescence intensity (MFI). This increase in MFI can lead to bead clusters merging and possible misidentification by FCAP Array™ software.

Additionally, background in large multiplexes and instrument performance issues can cause the FL3 intensity of the beads in the D and E rows to fluctuate, which results in populations merging or having increased peak CV that can cause similar bead clustering issues. If clustering fails for any reason, the entire data file is excluded from analysis by FCAP Array software. Therefore, samples should be diluted in order to keep PE signals within an acceptable range.

Note: This may cause samples with low analyte concentrations to become undetectable and expected concentration may be difficult to predict if using unknown samples.

When designing multiplex experiments, it may be necessary to analyze beads that are spatially close to each other in separate tubes, to prevent merging of adjacent clusters. This is most important with the beads in the D and E rows but it can extend to any bead that corresponds to an analyte that might be overly abundant in a particular sample.

BD FACSComp™ software is useful for setting up the flow cytometer, BD CellQuest™ software is required for acquiring samples, and FCAP Array software is required for subsequent data analysis of the BD CBA Flex Set assays.

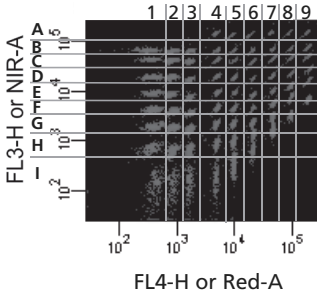


Figure 1

Preparation of Instrument Setup Beads

1. Label five 12 × 75 mm tubes *A, B, C, D,* and *E.*
2. Add 25 µL of PE Instrument Setup Bead F1 to tube D.
3. Add 50 µL of PE Positive Control Detector to tube D and vortex tube briefly to mix.
4. Incubate tube D for 15 minutes at room temperature, protect from light.
5. Add 25 µL of Instrument Setup Bead A9 to tube A.
6. Add 25 µL of Instrument Setup Bead A1 to tube B.
7. Add 25 µL of Instrument Setup Bead F9 to tube C.
8. Add 25 µL of Instrument Setup Bead F1 to tubes A, B, C, and D.
9. Add 50 µL of the mixed Capture Beads from the Flex Set experiment to tube E.

   Note: The mixed Capture Beads added in step 9 should be beads only and not contain PE detection reagent, standards, or sample.
10. Add 300 µL of Wash Buffer to tube D and vortex tube briefly to mix.
11. Add 350 µL of Wash Buffer to tubes A, B, C, and E. Vortex each tube briefly to mix.
12. Proceed to the next section.

Tube	Setup Beads
A	A9 + F1
B	A1 + F1
C	F9 + F1
D	PE-F1 + F1
E	Mixed Capture Beads

Instrument Setup with BD FACSComp™ Software and BD Calibrite™ Beads

1. Perform instrument startup.
2. Perform flow check.
3. Prepare tubes of BD Calibrite™ beads for a four-color setup.
4. Launch BD FACSComp™ software.
5. Run BD FACSComp software in Lyse/No Wash mode.

   Note: Time-delay calibration must be performed. For detailed information on using BD FACSComp with BD Calibrite beads to set up the flow cytometer, refer to the *BD FACSComp Software User's Guide* and the BD Calibrite Beads package insert.
6. Proceed to the next section.

Instrument Setup with Instrument Setup Beads

1. Launch BD CellQuest™ software and open the BD™ CBA Flex Set setup template.

   Note: The BD CBA Flex Set Setup Template file may be downloaded from **bdbiosciences.com/flexset**

2. Set the instrument to Acquisition mode.
3. Set SSC (side light scatter) and FSC (forward light scatter) to Log mode.
4. Decrease the SSC PMT voltage by 100 from what BD FACSComp set.
5. Set the Threshold to SSC at 650.
6. In Setup mode, run tube A. Follow the setup instructions on the following pages.

   Note: Pause and restart acquisition frequently during the instrument setup procedure to reset detected values after settings adjustments.

Adjust gate R1 so that the singlet bead population is located in gate R1 (***Figure 2a***).

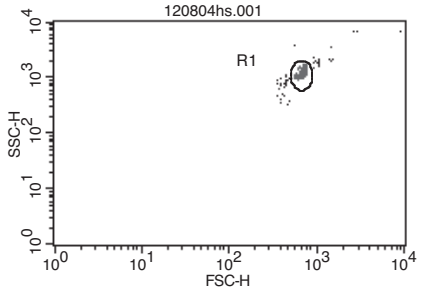
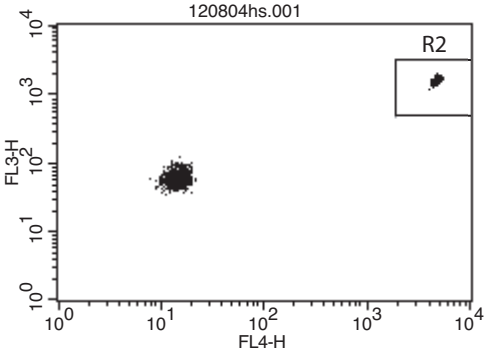


Figure 2a

Adjust the R2 gate so it gates the brightest bead population (***Figure 2b***). Adjust the FL3 PMT so that the FL3 median of the A9 bead population is around 1500 and then adjust the FL4 PMT so that the FL4 median of the A9 bead population is around 5000.



FL3 Median: 1512.47

FL4 Median: 5048.07

Figure 2b

Adjust gate R3 so the F1 bead population is inside it (***Figure 2c***). Adjust the FL2 PMT so the FL2 median of the F1 bead population is between 2.5 and 4.0. Proceed to the next page of the setup template.

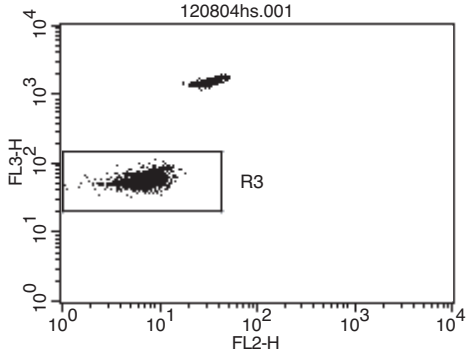


Figure 2c

Run tube B to adjust the compensation settings for FL4 – %FL3.

Adjust gate R4 so the A1 bead population (brightest bead in FL3 channel) is inside it (***Figure 2d***). Ensure that the left edge of gate R4 is touching the y-axis boundary of the dot plot. Adjust gate R5 so the F1 bead population is inside it (***Figure 2d***). Using the FL4 - %FL3 control, adjust the median of G4 until it is equal to the median of G5 (***Figure 2d***). Proceed to the next page of the setup template.

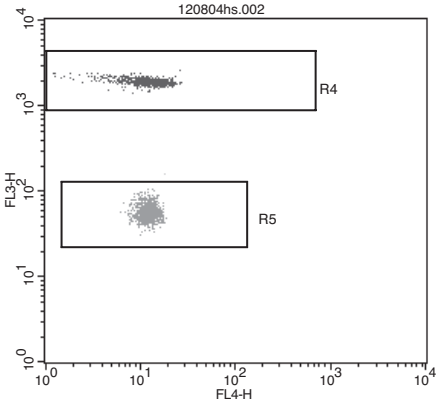


Figure 2d

Run tube C to adjust the compensation settings for FL3 – %FL4.

Adjust gate R6 so the F1 bead population (dimmest bead in FL4 channel) is inside it (***Figure 2e***). Adjust gate R7 so the F9 bead population is inside it (***Figure 2e***). Using the FL3 – %FL4 control, adjust the median of G7 until it is equal to the median of G6. Proceed to the next page of the setup template.

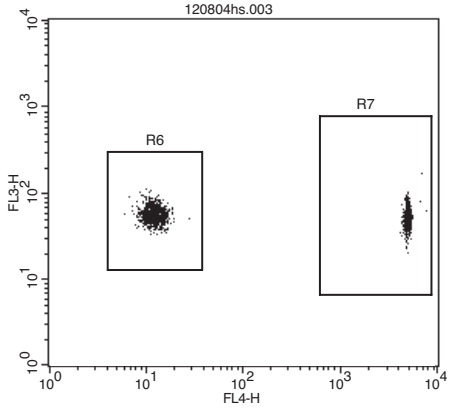


Figure 2e

Run tube D to adjust the compensation settings for FL3 – %FL2.

Adjust gate R8 so the F1 bead population (dimmest bead in FL2 channel) is inside it (***Figure 2f***). Adjust gate R9 so the PE-F1 bead population is inside it (***Figure 2f***). Using the FL3 – %FL2 control, adjust the median of G9 until it is equal to the median of G8. Proceed to the next page of the setup template.

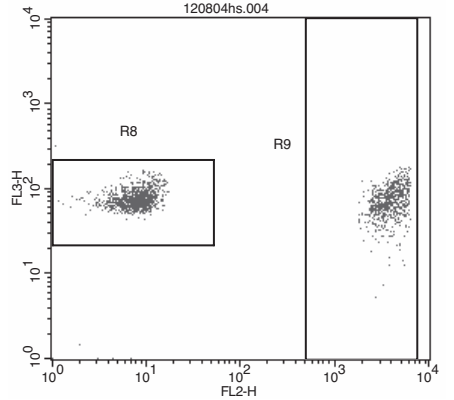
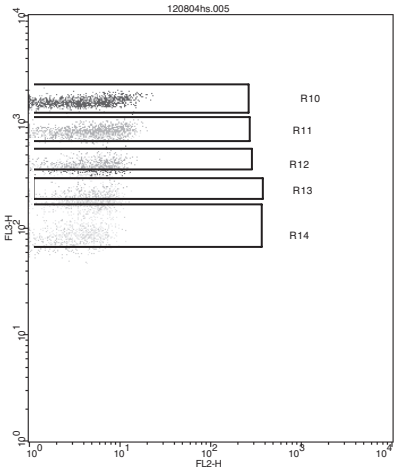


Figure 2f

Run tube E to adjust the compensation settings for FL2 – %FL3.

Adjust gates R10, R11, R12, R13, and R14 so each bead row falls within only one gate (see ***Figure 2g***). Ensure the left edge of each gate is touching the y-axis boundary of the dot plot. Using the FL2 – %FL3 control, adjust until the median of any row (G10, G11, G12, G13, or G14) equals 2 – 4 (***Figure 2g***).

Note: It is important not to overcompensate the bead populations in this step. No bead population should have a median below 2.



Gate G10 Median: 2.81

Gate G11 Median: 5.19

Gate G12 Median: 4.96

Gate G13 Median: 5.05

Gate G14 Median: 3.75

Figure 2g

Note: Not all populations shown in ***Figure 2g*** will be displayed in every setup; the populations displayed are dependent on the Flex Set beads used in the experiment.

Proceed to the next section.

Data Acquisition

1. Return to page 1 on the BD™ CBA Flex Set setup template.
2. Set acquisition mode.
3. In the Acquisition and Storage window, do the following:
   a. Set the Acquisition Gate to Accept G1=R1 events. (This will allow for only the events that fall into R1 to be saved).

   Note: Be sure that R1 is set correctly to avoid data loss.

   b. Set Collection Criteria for acquisition to stop at 300 × the number BD CBA Flex Set assays being used (eg, 300 × 6 = 1800 events for a 6 plex). This ensures that the sample file contains approximately 300 events of each bead population. Do not acquire more than 300 beads per population.
   c. Set Resolution to 1024.
   d. Click OK.

4. In setup mode, run tube number 1 and using the FSC vs. SSC dot plot, place the R1 region gate around the singlet bead population (see ***Figure 2a***).
5. Samples are now ready to be acquired.
6. Begin sample acquisition with the flow rate set to LO. Using the lowest flow rate can improve resolution of the individual bead populations in the bead plex.

To facilitate analysis of data files using the FCAP Array™ software and to avoid confusion, add a numeric suffix to each file that corresponds to the assay tube number (ie, Tube No. 1 containing 0 pg/ml could be saved as KT032598.001). The file name must be alphanumeric (ie, contain at least one letter).

Analysis of Sample Data

The analysis of BD™ CBA data is optimized when using FCAP Array software. Install the software according to the instructions in the *FCAP Array User's Guide*.

1. Transfer FCS data files for the experiment to the computer with the FCAP Array software.
2. Place all data files for a given experiment in a single folder.

Follow the instructions for creating an experiment and data analysis in the *FCAP Array Software User's Guide*.

Troubleshooting Tips

Problem	Suggested Solution
Debris (FSC/SSC) during sample acquisition	Increase the threshold or enable dual FSC and SSC thresholds. Set acquisiton to R1 events only to exclude debris. Use caution when setting these parameters as excluded events cannot be recovered post-acquisition.
Bead clusters merging	Be sure that setup beads are at the appropriate MFI targets as directed in the instrument setup procedure. If bottom standard or master bead mix tubes show merging populations instrument service may be required. Beads may merge in the FL3 orientation if sample concentrations are too high. Run daily instrument QC to ensure that instrument is performing well.
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 that the stopping rule, singlet gate, and thresholds are set correctly.
	Avoid aspiration of beads during wash step.

Notes

Notes

Notes

United States
877.232.8995

Canada
888.259.0187

Europe
32.53.720.550

Japan
0120.8555.90

Asia/Pacific
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