

# CBA data analysis with FCAP software

SOP014  
28/08/2009  
Wim Ammerlaan

## **Reference**

BD FCAP ArraySoftwareUser's Guide Windows Version Part No. 641489 Rev. A June 2006.

## **Introduction**

Cytometric Bead Array (CBA) data is most easily processed by using FCAP software. This program is specially designed for generating quick results from a CBA experiment.

The FCAP software recognizes clusters (= groups of beads with the same colour intensities) in the APC vs. APC-Cy7 coloured matrix of the CBA experiments. After assigning the proper analyte to the correct cluster, the program calculates the reporter fluorescence (PE) mean value of each cluster. With use of a standard curve, which is included in each experiment for each analyte, the program calculates the concentration of each analyte/multiplex component for each sample.

For analysing data, FCAP needs to be informed about the bead position in the matrix and the type of molecule on this bead of all multiplex components. This information can be found on the package insert of the analytes/antigens.

For processing data with FCAP the cytometer FCS files have to be exported from Diva in a FCS2.0 format. During this process it is possible to exclude debris, which reduces processing errors in FCAP.

FCAP produces a report with all the information of the experiment, including standard curves and analyte concentrations. Results from FCAP can be exported as a CSV file which can be imported in Excel for custom data processing.

## **Material**

- Diva 6.1.2 acquired experimental CBA files with standard range
- FCAP 1.0.1 software installed on ANA1
- Package inserts from the analytes

## **Method**

### **Exporting data from Diva software**

1. On Analyse station 1: Import the CBA experiment in Diva 6.1.2. The experiment should be located on the server "cfc on CRPnas\Canto data backup\BDExport\Experiment\XxX\XXyyymmdd bla bla

2. Open experiment (double click on experiment name) and check if gate P1 “singlets” in the FSC-A vs. SSC-A dotplot is placed correctly around the main population of the beads.
3. Right click on experiment name and Export FCS files.
4. In the dialogue box <Gate Events> select “Singlets” and <Version> tag FCS 2.0: <OK>
5. Export data to D:\BDexport\FCS\FCAP export <Save>
6. Diva generates in the FCAP export directory a subdirectory with the original experiment name where it saves the exported FCS files in FCS2.0 format.
7. While continuing the analysis leave the original experimental data in Diva accessible for eventual troubleshooting later.

### Analyse data in FCAP

1. FCAP array 1.0.1 is installed on Ana1. The Program runs only with a USB hardware lock (1 copy in house inserted in Ana1)
2. Double click on the FCAP icon on the desktop.  
User name: BD Biosciences  
Password: welcome
3. Start “New experiment with the wizard” (or click on the left top icon) and follow the instructions.
4. **Overview:** Nothing to enter; <Next>
5. **Test Samples:** In “Number of Samples” counter enter only the number of samples of the experiment to be analyzed. Standard range and Setup Control tubes should not be included; <Next> Check the result; <Next>
6. **Dilution and replicates:** In general nothing to enter; <Next>
7. **Selecting Saved Plex:** Click on the Plex name appropriate for this experiment. <Next>. If a new Plex needs to be created, because new analytes/antigens are included in your experiment, follow the procedure: “New Plex”, written in a sub protocol underneath this protocol.
8. **Plex Components:** Check if the components of the Plex chosen are identical to the analytes in your experiment. Confirm the bead ID (position in the matrix) with the information on the analyte package insert. <Next> Catalog and lot numbers are in general not used.
9. **Clustering parameters:**
  - a. Click <Load Data File> and locate in D:/BD Export/FCS/FCAP export the experiment of interest. <Select>;
  - b. Click on sample with “20pg ,2f,ml\_singlets” <Select>
  - c. Instrument name: “BD FACS Canto”
  - d. Scatter parameter: “ FSC-A”
  - e. Number of scatter peaks: “1”
  - f. Clustering parameters: “APC-A” and “APC-Cy7-A”
  - g. Reporter parameter: “PE-A”
  - h. Clustering has succeeded <Next> Clustering failed: See trouble shooting underneath.
10. **Analyte Assignment:** Confirm if the cluster ID is placed at the correct Bead ID. Rows of bead clusters are designed A to E and the columns 4 to 9. The Bead ID name should help you locating the associated cluster. When all

- clusters are correctly linked to an analyte click <Next>. If an error occurs click <Clear Assignment> and create a new correct assignment.
11. **Qualitative/Quantitative:** Tag Quantitative for concentration determination; for Fitting equation, click on the white line/space and select “4 parameter logistics “. Tag qualitative if only a positive or negative score is required. In general quantitative analysis is requested. <Next>
  12. **Standards:**
    - a. Type the number of standards samples in the counter including the “0” tube. In general: “12”
    - b. Select the Unit. In general: “pg/ml”
    - c. Type for each standard tube the concentration of analyte added, starting with zero going upwards.
    - d. Tag uniform concentrations for all analytes
    - e. Number of replicates:”1”
    - f. <Next>
  13. **Controls:** Nothing to enter; <Next>
  14. **Reporting Messages:** Nothing to enter; <Next>
  15. **Plate Layout Options:** Tag
    - a. 96 well plate
    - b. Row by Row
    - c. Place samples at the end
    - d. Standards / Samples start on a new row / column
    - e. <Next>
  16. **Experiment Name:** Type the experiment name in the empty box. Please use the following structure XXyymmdd CBA, where XX are your initials, and the yymmdd is the inverse date of data acquisition. <Finish>
  17. **Save As:** Save the data in a new folder named XXyymmdd CBA in “My documents”. If you have made a new Plex, Tag the Save Plex box and name the Plex using the following structure XXyymmdd x beads.<Finish>
  18. **File Assignment:**
    - a. Click on the worksheet < File Assignment>
    - b. In the left panel are listed first the standards (step 12) followed by the number of samples (step 5). In the right panel are the Diva exported FCS2.0 singlets files from D:/BDExport/FCS/FCAP export/XXyymmdd CBA .
    - c. Click/Highlight the 0 pg standard tube file in the right panel.
    - d. Click/Highlight the associated Std01/1 file in the left panel
    - e. Click on the left orientated arrow head in the central vertical bar bewt to assign the two tubes
    - f. Continue in the same way for all standards followed by all samples
    - g. (If all standards and samples are correctly listed you could use the double left orientated arrow head to link all tubes in one click )
    - h. Check carefully if all tubes are correctly assigned.
  19. **Calculate:** Click on the green calculator in the icon bar to perform all calculation
  20. **Analysis messages:** To store the error messages
    - a. Click on button “Copy Results to Clipboard”
    - b. Open MS Word
    - c. Paste in a word file the error messages from clipboard

- d. Save file as XXyymmdd CBA error.doc in the folder generated at step 17 **Save As**
  - e. Click <OK>
21. **Report Printout:**
- a. Click on the worksheet <Report Printout>
  - b. File/Print report to printer and to pdf,
  - c. Save pdf file as XXyymmdd CBA result.pdf in the folder generated at step 17 **Save As**
22. **Raw Data:**
- a. Click on the worksheet <Raw Data>
  - b. Click on <Export> at the bottom of the screen
  - c. Tag everything **except** <controls> and <Analytes in rows, samples in columns>
  - d. <Continue>
  - e. Save the raw data file as XXyymmdd CBA stat.csv in the folder generated at step 17 **Save As**.
23. **Exit:** File/exit FCAP, Save before closing <Yes>
24. **Data export:** All reports and prints are generated. Copy the folder with all the files from “My Documents” to a space which is accessible from your office computer.

### Sub protocol: New Plex

When your CBA experiment contains new analytes, you are obliged to create a new Plex. This can be done in:

- Protocol: The FCAP Array Experiment Wizard at step 7: Selecting a Saved Plex and adjust this to the new beads content
- Saved Plex library (blue icon in the icon bar, Plex 1, 2, 3).
- FCAP Array New Plex Wizard.(icon with magic stick and Plex paper)

Here follows the first option: “The FCAP Array Experiment Wizard at step 7”

1. Select “New Plex” <Next>
2. Check in the Beads panel on the left side, underneath the All Beads subheading if the analytes of your Plex are already entered. If not click <Edit>
  - a. At the Beads Information panel on the right type the requested information which you can find on the package insert of each analyte. (catalog, lot and barcode are not essential) Click <Add>
  - b. Repeat step 2a for all new Analytes. <OK>
3. The left panel is the bead library which displays all the bead information stored in FCAP. In the bead panel scroll down to “All beads” Select all the beads used in the experiment with use of shift and control keys
4. Click on the right orientated arrowhead between the 2 panels; all the beads used in your CBA should be displayed in the selected beads panel. < Next>
5. **Clustering parameters:**
  - a. Click <Load Data File> and locate in D:/BD Export/FCS/FCAP export the experiment of interest. <Select>;
  - b. Click on sample with “20pg ,2f,ml\_singlets” <Select>
  - c. Instrument name: “BD FACS Canto”

- d. Scatter parameter: “FSC-A”
  - e. Number of scatter peaks: “1”
  - f. Clustering parameters: “APC-A” and “APC-Cy7-A”
  - g. Reporter parameter: “PE-A”
  - h. Clustering has succeeded <Next> Clustering failed: See trouble shooting underneath.
6. **Analyte Assignment:** Assign an analyte to a cluster by selecting a Cluster ID in the left panel and double click on the corresponding cluster in the APC vs. APC-Cy7 dot plot. When all analytes are assigned to the correct cluster <Next>
  7. Continue the Protocol: “The FCAP Array Experiment Wizard” until **Analyse data in FCAP** step 17 Save As. Tag the Save Plex box and name the Plex using the following structure XXyymmdd x beads.<Finish>

## Trouble shooting

*Singlets population in FSC vs. SSC dot plot consist of distinct groups of beads.*

Sometimes beads or different batches of beads have variable scatter properties. In the bead mixture this shows up as distinct groups in the FSC vs. SSC dot plot. Confirm these additional populations are not doublets in the SSC-A vs. SSC-H dot plot. If the various groups are indeed Singlets, select them all in the Singlet gate and export as FCS2.0 file. Follow the protocol Analyse data in FCAP as written above until step 9 Load data file. Adjust the number of scatter peaks until all clusters are selected. Continue with the Analyse data in FCAP protocol.

*Less than 60 beads in one of the Plex components*

If for any reason one of the beads in the bead mix is far less than the others it could cause troubles with the clustering and this could block the complete data analysis. The troublesome population should be excluded from analysis.

In Diva create a 2<sup>nd</sup> gate (p2) in the APC-A vs. APC-Cy7-A dot plot. Include all bead clusters except the one with lower bead count. Export FCS files Singlets and P2 in FCS2.0 format.

In FCAP create a new Plex without the troublesome bead cluster and perform the data analysis is described above.

*Critical error in one standard tube*

If for any reason one of the standard tubes cause a serious error (e.g. clog during data acquisition) the standard curve cannot be made and no concentrations of the samples are calculated.

At step 12 generate a standard range without the troublesome standard tube. Continue the Analyse data in FCAP, until step 18 File Assignment. Do not assign the troublesome standard tube in the right panel to a corresponding file in the left panel. There should not be a file in the left panel with the same concentrations. Leave the file untagged in the right panel and continue with the calculations.

*Human TH1/TH2 CBA kit looks very different.*

The matrix of this CBA is based on FITC vs. APC. Most likely this CBA originates from a time where not many flowcytometers had an APC Cy7 Parameter.

This requires a different Canto setup and FCAP data analysis.

At the Canto load the MR090611 CBA Th1 Th2 template for data acquisition.

At FCAP follow the conventional protocol until step 9 Load data file, select

Clustering parameters: "FITC-A" and "APC"

The rest remains the same

### *Clustering failed*

Several reasons can cause the clustering to fail.

Most frequent is dirt in the tube. By making the singlets gate very tight around the beads, a maximum of debris and dirt can be excluded from data analysis.

When debris covers a part of matrix, this part can be excluded by creating a 2<sup>nd</sup> gate (See *Less than 60 beads in one of the Plex components*)

A cluster is not recognized one group because different batches of one analyte were used. Try to gate out in Diva in the APC-A vs. APC-Cy7 dot plot the minor population of this cluster with the 2<sup>nd</sup> gate.

Wrong Plex was chosen. Restart the analysis with the correct Plex.