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Application Note

Quantification of Cytokines Using BD™ Cytometric Bead Array on the BD™ FACSVerse System and Analysis in FCAP Array™ Software

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Summary

Characterization of cytokines from various T-cell subsets, in response to physiological immunomodulators, is important for researchers investigating basic immunological function and disease pathogenesis. Th17, a recently characterized T-cell subset, is of particular interest in T-cell biology because of its plasticity and its ability to change phenotype in response to its environment. The BD™ Cytometric Bead Array (CBA) Human Th1/Th2/Th17 Kit is an essential tool for research in T-cell biology. It contains reagents for measuring particular sets of cytokines using flow cytometry to determine if plasticity toward a Th1, Th2, or Th17 response may be occurring during T-cell differentiation. The BD FACSVerse™ system includes the cytometer and BD FACSuite™ software for acquisition and analysis. Designed for use with BD CBA Kits and Flex Sets, pre-defined experiment templates are available in BD FACSuite which provide optimal settings for acquisition of CBA data. Further, the data files from BD FACSuite can be exported and analyzed by FCAP Array™ v3.0 software which provides a robust and flexible analysis solution for quantification of cytokines. The BD CBA Flex Set system enables researchers to multiplex custom cytokine panels to study T-cell plasticity. This application note demonstrates a protocol for the stimulation of peripheral blood mononuclear cells (PBMCs) using phorbol myristate acetate (PMA) and ionomycin. Cytokines produced after stimulation were analyzed using a BD CBA Th1/Th2/Th17 CBA Kit on a BD FACSVerse system followed by analysis in FCAP Array software. A BD Flex Set 8-plex was also used to characterize the cytokine profile of stimulated PBMCs. The 8-plex was acquired on a BD FACSVerse analyzer and the data was analyzed in FCAP Array software. BD CBA Kits and BD Flex Sets, when used in combination with the BD FACSVerse system, BD FACSuite software, and FCAP Array software, can provide a comprehensive tool for analysis of T-cell plasticity.

Introduction

Cytokines are soluble proteins that play an important role in immunity, inflammation, and hematopoiesis. They are rapidly produced by a variety of cell types and secreted in response to specific and non-specific stimuli. T lymphocytes play an important role in the regulation of the immune system by secreting key cytokines that can drive T-cell differentiation into several characteristic subsets based on the stimuli. These T-cell subsets have characteristic cytokine profiles and perform different functions. T cells can be categorized into Th1, Th2, or Th17 subsets depending upon the cytokines they produce.[1] For example, Th1 cells produce interferon gamma (IFN-γ), interleukin (IL)-2, and tumor necrosis factor (TNF). Th1 cells orchestrate cell-mediated immune responses and help eliminate intracellular pathogens such as viruses. Th2 cells produce IL-4, IL-5, IL-6, IL-10, and IL-13. These cells regulate humoral immune responses against extracellular infections such as bacteria and parasitic organisms. Th17 cells provide immunity to extracellular bacteria and fungi, and are characterized by the production of IL-17A, IL-17F, IL-21, IL-22, IL-26, TNF, and chemokine (C-C motif) ligand 20 (CCL20). T regulatory (Treg) cells and Th9 cells have also been characterized based on their secretion of cytokines. For further details about T-cell biology, visit **bdbiosciences.com/tcell**.

BD CBA Reagents

To characterize T cell cytokine profiles, cytokines secreted into culture media or supernatants can be quantified by techniques such as BD CBA, ELISA, and Western blot. BD CBA can quantify multiple cytokines from the same sample simultaneously, while ELISA is useful for measuring levels of single cytokines. The BD CBA system uses the broad dynamic range of fluorescence detection offered by flow cytometry and antibody-coated beads to efficiently capture analytes such as cytokines. Each bead in the array has a unique fluorescence intensity so that beads can be mixed and acquired simultaneously in a single tube. Figure 1 outlines the basic principle of the BD CBA assay. This method significantly reduces sample volume requirements and time to results in comparison with traditional ELISA and Western blot techniques.

The BD CBA portfolio includes assays for the measurement of a variety of soluble and intracellular proteins, including cytokines, chemokines, growth factors, and phosphorylated cell signaling proteins. BD CBA solutions are available in two formats to meet the diverse needs of researchers:

• BD CBA Kits are preconfigured for achieving consistent results for routine panels.

• BD CBA Flex Sets provide an open and configurable method of detection so that researchers can build their own multiplex assays.
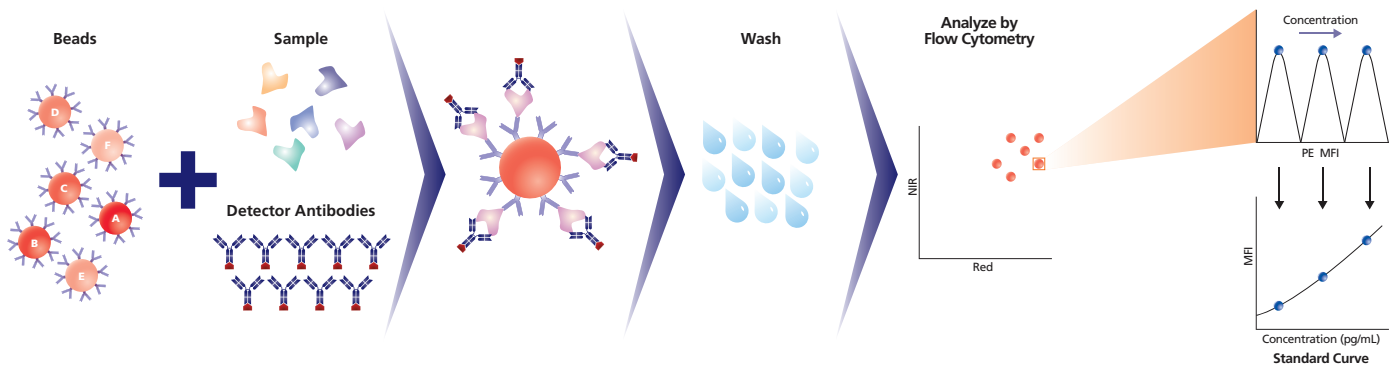
Figure 1. Overview of the BD CBA assay.

The BD CBA Human Th1/Th2/Th17 Kit is an essential tool for research in T-cell biology. This kit contains reagents for measuring a particular set of cytokines associated with the Th1, Th2, and Th17 response. The kit contains reagents to measure IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ, and TNF. Many of these cytokines are multifunctional and are involved in proliferation (IL-2) and regulation (IL-10) of multiple T-cell differentiation pathways. Others are key cytokines unique to particular differentiation/polarization pathways. Overall, the cytokine profile generated by this kit can help determine the T-cell polarization towards Th1, Th2, or Th17.

In addition to the CBA Kits, BD CBA Flex Sets provide flexibility to expand upon the analytes in the preconfigured Th1/Th2/Th17 Kit. The CBA Flex Sets are formulated for combination into any plex size, from a single plex up to a 30 plex, to maximize assay flexibility. This allows researchers to multiplex custom cytokines for studying T-cell biology.

BD FACSVerse System and BD FACSuite Software

The BD FACSVerse system is a high-performance flow cytometer that incorporates easy-to-use, task-based workflows. The system streamlines every stage of operation from automated setup through data analysis. The system includes automated procedures for setting up the instrument and assays, and configurable user interfaces that provide maximum usability for researchers. These functions are integrated to provide simplified use for routine applications while simultaneously providing more powerful acquisition and analysis tools for more complex applications. In addition, the system can be upgraded with the BD FACS™ Universal Loader option (the Loader), which enables use of either tubes or plates for samples, with or without barcoding for sample identification and tracking.

To provide ease of acquisition of CBA standards and samples, BD FACSVerse experiment templates for CBA Kits and Flex Sets have been made available at **bdbiosciences.com/cbasetup** for download. These experiments contain pre-defined tube settings for acquisition of CBA standards and samples which facilitate acquisition on a BD FACSVerse cytometer. The experiment templates also contain CBA-specific keywords, which further provide ease of analysis of CBA data in FCAP Array software. Using these experiment templates, CBA assays can be created in BD FACSuite software and then run in a worklist on the Loader in a high-throughput manner. After acquisition, flow cytometry standard (FCS) files are exported and data analysis can be performed in FCAP Array software. Figure 2 outlines the CBA workflow for acquisition and analysis.
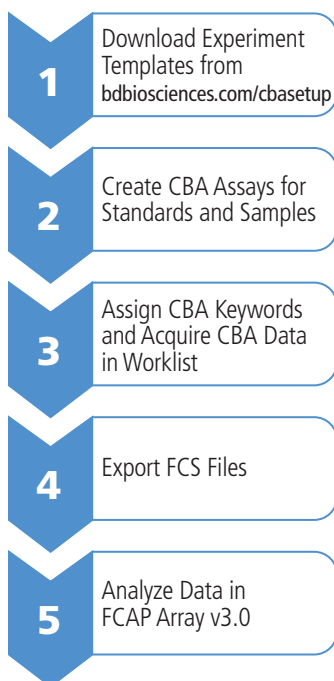
FCAP Array Software

FCAP Array v3.0 software facilitates the data analysis of CBA Kits and Flex Sets. The software performs quantitative analysis of CBA data and determines multiple analyte concentrations in test samples based on concentrations of standards. The software reads the FCS data files, locates clusters (to which analytes have been assigned), and then determines the median fluorescent intensity (MFI) of the detector antibody for each analyte. The software fits a standard curve to the data based on the concentration of standards. Various curve-fitting models are available for calculating the concentration of the measured analytes in a sample.

When used in conjunction with BD FACSVerse analyzer and BD FACSuite software, the BD CBA system provides a quick and easy system for acquisition of BD CBA experiments and subsequent analysis using FCAP Array software. When using the BD FACSuite workflow within FCAP Array, keywords assigned during acquisition are automatically read by FCAP Array in order to minimize the user input required to analyze the data and decrease the time to results.

1 Download Experiment Templates from bdbiosciences.com/cbasetup

2 Create CBA Assays for Standards and Samples

3 Assign CBA Keywords and Acquire CBA Data in Worklist

4 Export FCS Files

5 Analyze Data in FCAP Array v3.0

Figure 2. Overview of CBA workflow.

Objective

The objective of this application note is to demonstrate the ease of use of acquisition of BD CBA Kits and Flex Sets data on the BD FACSVerse system with BD FACSuite software, and subsequent analysis in FCAP Array software. A proof-of-principle protocol for secretion of cytokines from human PBMCs stimulated with PMA and ionomycin was used to demonstrate BD CBA Th1/Th2/Th17 Kit and a BD Flex Set on the BD FACSVerse system.

Methods

BD CBA Kits and Flex Sets

Product Description	Vendor	Catalog Number
Human Th1/Th2/Th17 Kit	BD Biosciences	560484
Human IFN-γ Flex Set (Bead E7)	BD Biosciences	558269
Human IL-2 Flex Set (Bead A4)	BD Biosciences	558270
Human IL-4 Flex Set (Bead A5)	BD Biosciences	558272
Human IL-5 Flex Set (Bead A6)	BD Biosciences	558278
Human IL-6 Flex Set (Bead A7)	BD Biosciences	558276
Human IL-10 Flex Set (Bead B7)	BD Biosciences	558274
Human IL-17A Flex Set (Bead B5)	BD Biosciences	560383
Human TNF Flex Set (Bead D9)	BD Biosciences	558273
Human Soluble Protein Master Buffer Kit	BD Biosciences	558264 (100 tests) 558265 (500 tests)
IL-2, Recombinant Human (rhIL-2)	BD Biosciences	554603
IL-4, Recombinant Human (rhIL-4)	BD Biosciences	554605

Antibodies

Specificity	Clone	Format	Isotype	Vendor	Catalog Number
CD3	UCHT1	NA/LE	Ms IgG$_1$, κ	BD Biosciences	555329
CD28	CD28.2	NA/LE	Ms IgG$_1$, κ	BD Biosciences	555725

Reagents and Materials

Product Description	Vendor	Catalog Number
BD Vacutainer® Tubes with Sodium Heparin	BD Medical	367874
BD Falcon™ 6-Well Cell Culture Plate	BD Biosciences	353934
BD Falcon Cell Culture Flasks 75 cm^2	BD Biosciences	353110
BD FACSuite™ CS&T research beads	BD Biosciences	650621 (50 Tests) 650622 (150 Tests)
BD FACSuite FC Beads - 4c Research Kit	BD Biosciences	650625

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BD FACSVerse Instrument Configuration

Wavelength (nm)	Detector	Dichroic Mirror (nm)	Bandpass Filter (nm)	Fluorochromes for BD CBA Kits	Fluorochromes for BD CBA Flex Sets
488	D	560 LP	586/42	PE	PE
640	A	752 LP	783/56	N/A	CBA NIR (APC-Cy™7)
	B	660/10 BP	660/10	CBA Red (APC)	CBA Red (APC)

Software

Product Description	Vendor	Catalog Number
FCAP Array v3.0 Software	BD Biosciences	652099

Specimens

Blood specimens were collected from donors who consented to participate in an Institutional Review Board–approved protocol.

Methods

Generation of Cytokine-Containing Supernatants
The following protocol was used to stimulate PBMCs to activate human T lymphocytes:

Coating the Plates
Two hundred microliters of anti-CD3 (10 µg/mL) was added to each well of a 6-well plate and incubated for 18–24 hours at 4°C.

Isolation of Human PBMCs
1. Whole blood was collected from normal donors into BD Vacutainer tubes (heparin) and diluted 1:1 with sterile Dulbecco's Phosphate-Buffered Saline (DPBS) (Gibco, No. 14190) at room temperature (RT).

2. PBMCs were isolated from the whole blood using Ficoll-Paque™ PLUS (GE Healthcare, No. 17-1440-02) according to the technical data sheet (TDS).[2]

3. Isolated cells were washed in DPBS (225g, 10 min, RT) and resuspended in 10 mL of Complete Medium [RPMI 1640 (Hyclone, No. SH30096.02) + 10% fetal bovine serum (FBS) (Hyclone, No. SH30151.03) + 1% L-glutamine and 1% penicillin and streptomycin (Gibco, No.10378)].

4. Cell concentration and viability were determined using the Trypan blue dye exclusion method and PBMCs were suspended at a concentration of 5×10^5 cells/mL in 36 mL of Complete Medium.

Stimulation of PBMCs
1. The following stimulants were added to the 36-mL cell suspension.

Stimulant	Final concentration
Anti-CD28	2 µg/mL
rhIL-2	10 ng/mL
rhIL-4	50 ng/mL

2. The 200 µL of CD3 antibody was removed from the 6-well plate (coated above), and each well was rinsed once with Complete Medium.

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3. Six milliliters of cell suspension–containing stimulants (above) were added to each well of an anti-CD3–coated plate (total of 6 wells) and incubated for 2 days at 37°C (5% CO_2 in air).

4. After 2 days, the cells were harvested from each well, pooled into 50-mL conical tubes, and centrifuged (225g, 5 min, RT).

5. The supernatant was removed and the cells were resuspended at 5×10^5 cells/mL in Complete Medium containing 10 ng/mL of IL-2 and 50 ng/mL of IL-4 in a T-75 tissue culture flask (36 mL/flask).

6. The cells were cultured for an additional 2 days (37°C, 5% CO_2 in air).

7. After 2 days, the cells were harvested and washed twice with 10 mL of Complete Medium (225g, 10 min, RT).

8. The cells were resuspended at a concentration of 5×10^5 cells/mL in 36 mL of Complete Medium containing 5 ng/mL of PMA (Sigma, No. P8139) and 500 ng/mL of ionomycin (Sigma, No. I0634).

9. The cells were incubated for 4 hours (37°C, 5% CO_2 in air).

10. The cell suspension was centrifuged (225g, 10 min, RT), and the supernatants were harvested and dispensed into 0.5-mL aliquots in microcentrifuge tubes. An aliquot was immediately tested for cytokines using a CBA assay and the rest of the aliquots were frozen at –80°C until analyzed.

CBA Protocol

Supernatants prepared as described in the previous section were thawed and tested for the presence of IL-2, IL-4, IL-5, IL-6, IL-10, IL-17A, TNF, and IFN-γ using either a CBA Th1/Th2/Th17 Kit or CBA Flex Set following the protocols described in the *BD CBA Human Th1/Th2/Th17 Kit Instruction Manual*[3] and the *Human Soluble Protein Master Buffer Kit Instruction Manual*,[4] respectively.

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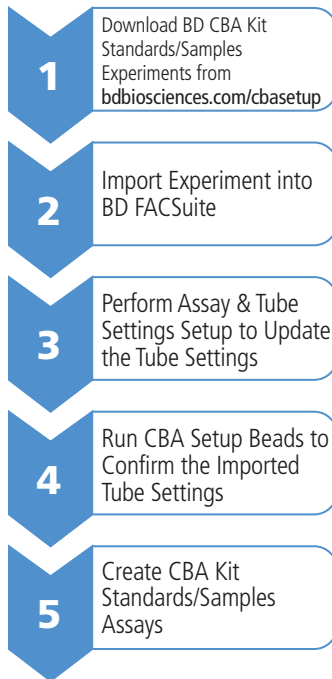
Figure 3. BD CBA Kit Initial Setup Workflow.
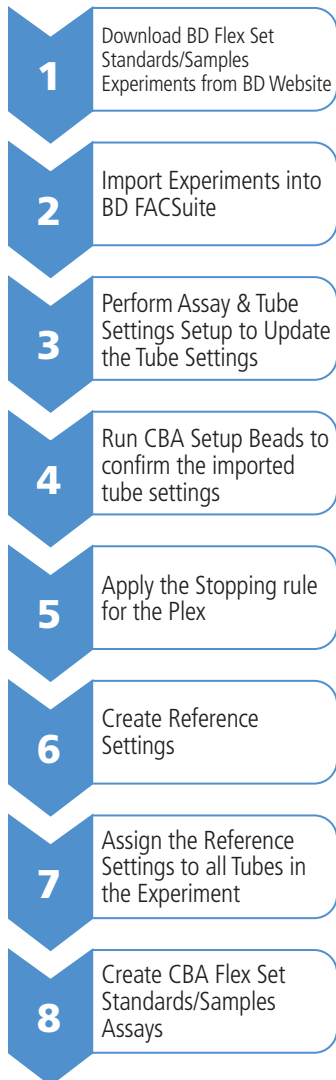
BD CBA Kit Assay for Standards and Samples

To run samples automatically in a worklist on the Loader, CBA assays for standards and samples were created in BD FACSuite software. Figure 3 shows the steps for creating a CBA assay. The CBA Kit standards and samples experiments were downloaded and imported into BD FACSuite following the instructions in the *Guide to Using BD FACSuite™ Software with BD™ CBA Products*.[5] Performance quality control (QC) was performed using BD FACSuite CS&T beads as outlined in the *BD FACSVerse System User's Guide*.[6] Assay and tube settings setup was also performed using BD FACSuite CS&T beads as outlined in the *BD FACSVerse System User's Guide*.[6] The imported tube settings were then confirmed by opening the Th1/Th2/Th17 CBA Kit standards experiment and verified that the CBA Setup Beads fell in the proper locations (Figure 4). Once the tube settings were confirmed, an assay for the acquisition of CBA Kit standards was created. Following the same steps, a similar assay for the acquisition of CBA Kit samples was also created.
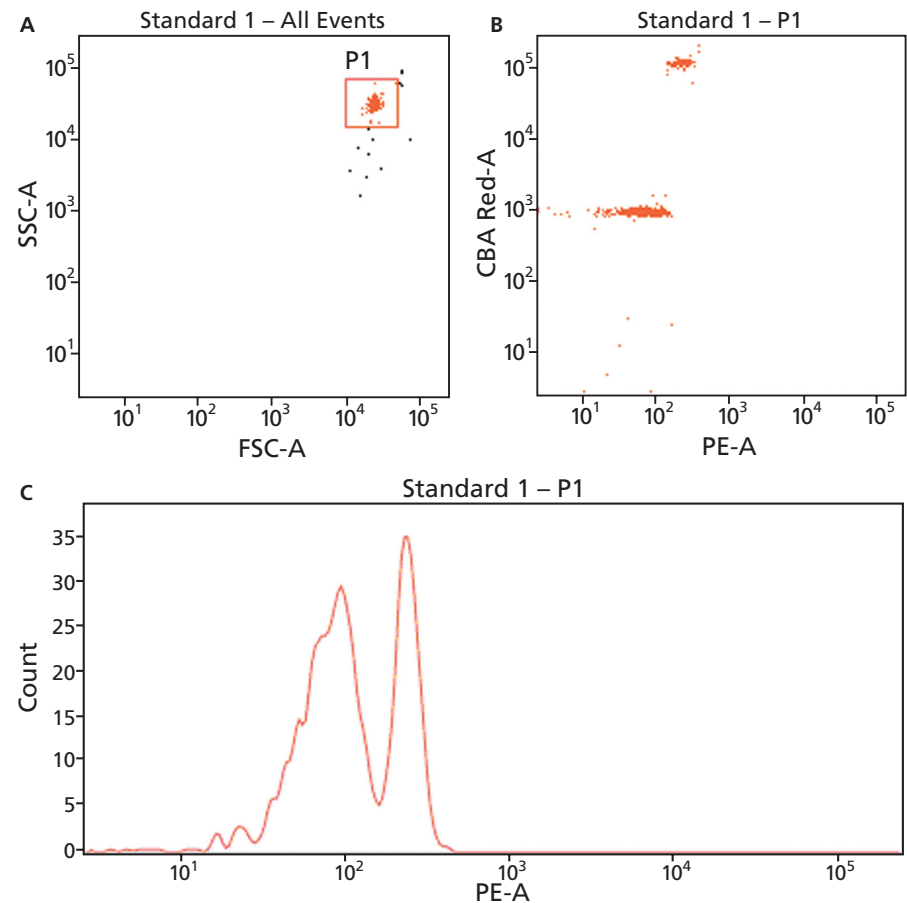
Figure 4. BD CBA Kit setup using CBA beads.
A) FSC-A vs SSC-A plot showing bead singlets within the P1 gate. B) PE-A vs CBA Red-A plot showing the bright bead in the CBA Red channel adjusted to 10^5. C) PE-A histogram showing the beads adjusted between 10^1 and 10^3 in the PE channel.

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1 Download BD Flex Set Standards/Samples Experiments from BD Website

2 Import Experiments into BD FACSuite

3 Perform Assay & Tube Settings Setup to Update the Tube Settings

4 Run CBA Setup Beads to confirm the imported tube settings

5 Apply the Stopping rule for the Plex

6 Create Reference Settings

7 Assign the Reference Settings to all Tubes in the Experiment

8 Create CBA Flex Set Standards/Samples Assays

Figure 5. BD CBA Flex Set Initial Setup Workflow.

BD CBA Flex Set Assay for Standards and Samples

Figure 5 outlines the workflow for creating the CBA Flex Set assays for standards and samples in BD FACSuite software. The Flex Set standards and sample experiments were downloaded and imported into BD FACSuite following the instructions in the *Guide to Using BD FACSuite™ Software with BD™ CBA Products*.[5] Performance QC was performed using BD FACSuite CS&T beads as outlined in the *BD FACSVerse System Users Guide*.[6] Assay and tube settings setup was performed after selecting the tube settings that were imported as part of the experiments. Using the Flex Set Standards experiment, CBA Setup Beads were previewed to verify that the beads fell in the proper locations (Figure 6). A stopping rule of 300 events per analyte was added (2,400 events for 8-plex). Reference settings for compensation were created using the CBA Setup Beads and then applied to each tube in the standards experiment. Finally, an assay for the acquisition of CBA Flex Set standards was created. Following the same steps, an assay for the acquisition of CBA Flex Set samples was also created. For details about creating reference settings, follow the instructions in the *Guide to Using BD FACSuite™ Software with BD™ CBA Products*.[5]
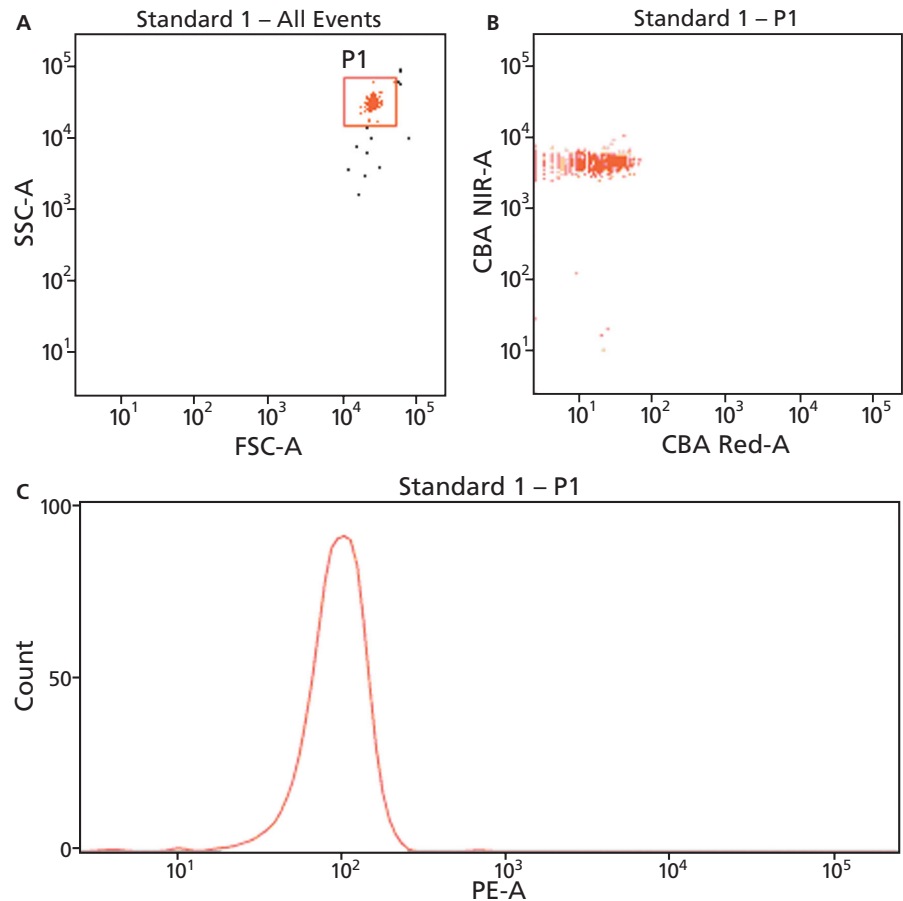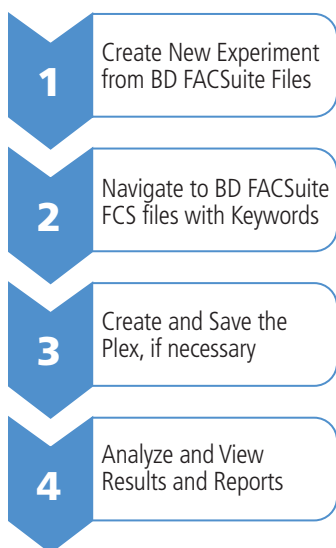
Figure 6. BD CBA Flex Set setup using CBA beads.
A) FSC-A vs SSC-A plot showing bead singlets within the P1 gate. B) CBA Red-A vs CBA NIR-A plot showing the mean of 160,000 ±2,000 for CBA Red and CBA NIR. C) PE-A histogram showing the beads adjusted between 10^1 and 10^3 in the PE channel.

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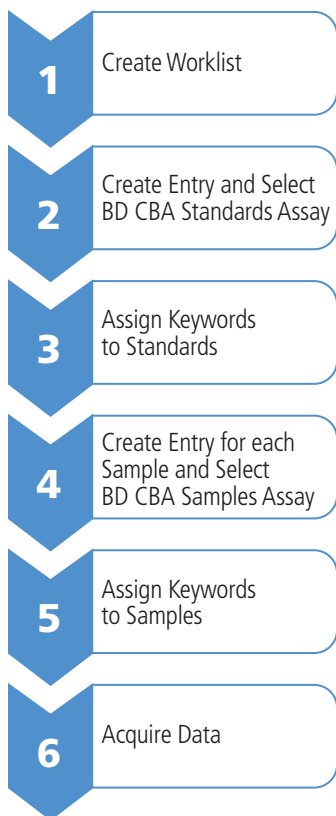
Figure 7. Typical CBA Acquisition Workflow.

1. Create Worklist
2. Create Entry and Select BD CBA Standards Assay
3. Assign Keywords to Standards
4. Create Entry for each Sample and Select BD CBA Samples Assay
5. Assign Keywords to Samples
6. Acquire Data

Figure 8. Data Analysis Workflow in FCAP Array v3.0.

1. Create New Experiment from BD FACSuite Files
2. Navigate to BD FACSuite FCS files with Keywords
3. Create and Save the Plex, if necessary
4. Analyze and View Results and Reports

Acquisition of CBA Kits and Flex Set Standards and Samples

After creating the CBA Kit and Flex Set assays for standards and samples, they were used in the worklist and acquired on a BD FACSVerse system using the Loader. The workflow for acquisition is shown in Figure 7. For details about creating worklists, follow the instructions in the *BD FACSVerse System User's Guide*.[6]
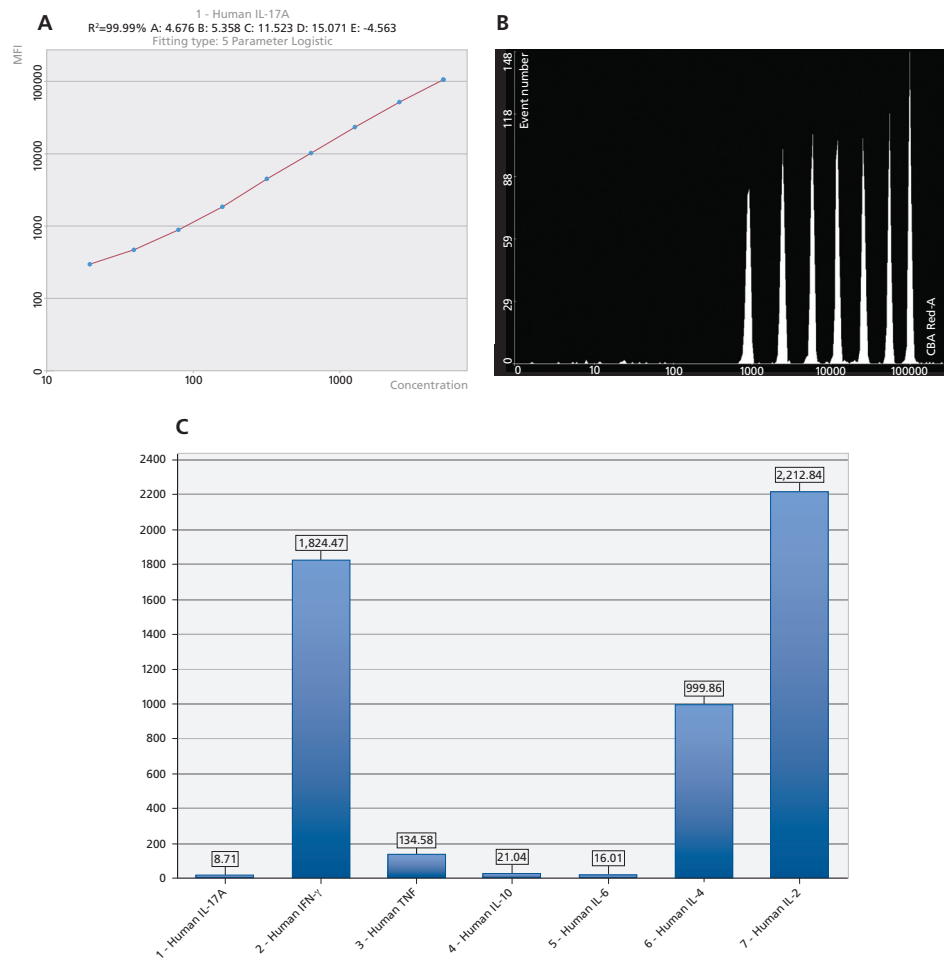
Data Analysis

After acquisition of the CBA Kit/Flex Set data in BD FACSuite software, the FCS files were exported for further analysis in FCAP Array v3.0 software. The basic workflow for analyzing data in FCAP Array is presented in Figure 8. A new experiment was created with an option to open BD FACSuite files following the instructions in the *FCAP Array Software Version 3.0 User's Guide*.[7] Based on the system keywords assigned during the acquisition, the software automatically identified the BD FACSuite files and mapped the plate locations correctly. Further, the keywords also enabled FCAP Array to differentiate the standards from the samples, and to calculate the dilution factor. For data analysis, each plex template was initially created in FCAP Array software which contained information about standards, beads, analytes, and instrument settings. For details about working with plexes, see the *FCAP Array Software Version 3.0 User's Guide*.[7]

Results and Discussion

The BD CBA Human Th1/Th2/Th17 Kit is an essential tool for research in T-cell biology. It contains reagents to quantify IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A using flow cytometry to determine if plasticity may be occurring toward a Th1, Th2, or Th17 response. A proof-of-principle protocol to stimulate cytokines in activated T cells was used and the cytokines (IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A) were quantified using Th1/Th2/Th17 Kit. In addition, BD CBA Flex Set was also used to expand upon the analytes that were measured in the pre-configured Th1/Th2/Th17 Kit, which allowed creation of a customized multiplex panel to study T-cell plasticity.

Quantification of Cytokine Levels Using the BD CBA Th1/Th2/Th17 Kit

The cytokine response from activated T cells was quantified using a BD CBA Th1/Th2/Th17 Kit. The data was acquired on the BD FACSVerse system using CBA sample and standards assays in a worklist. The FCS files were exported and data was analyzed in FCAP Array software (Figure 9).

Figure 9. Analysis of BD CBA Th1/Th2/Th17 Kit data in FCAP Array.
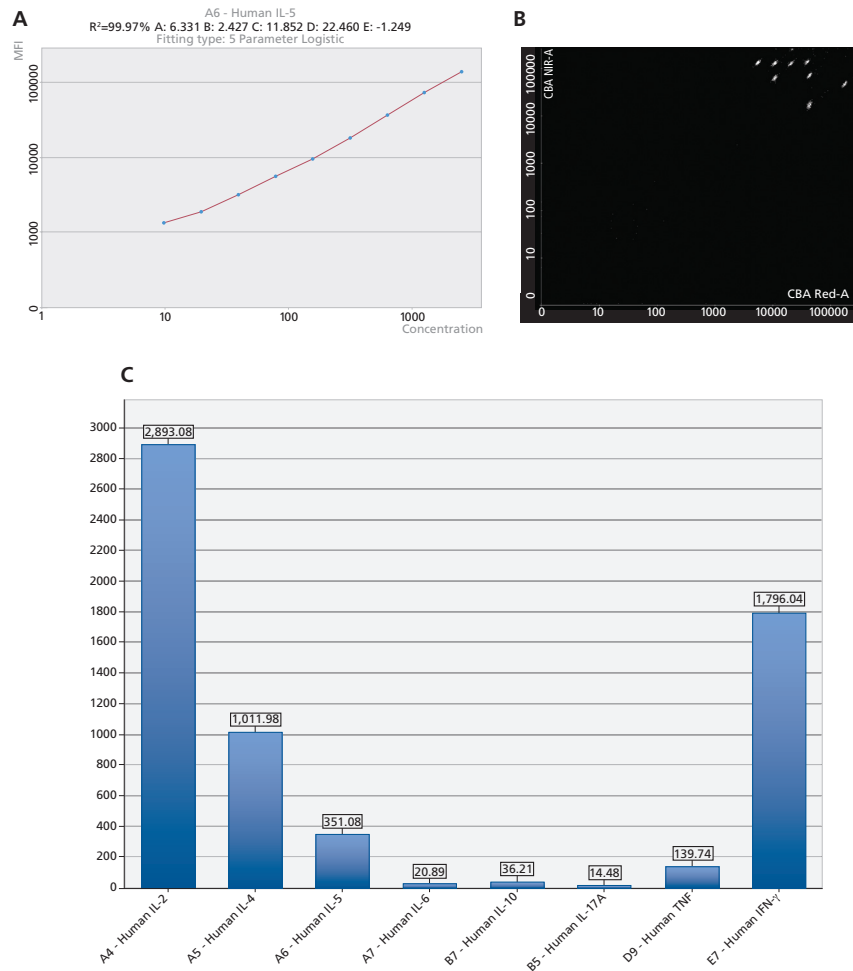*Panel A. Standard curve of IL-17A.*
Panel B. Histogram of CBA Red-A showing seven bead populations with distinct fluorescence intensities.
Panel C. Bar graph showing concentrations of all the cytokines (pg/mL for 1:16 dilution of sample).

Quantification of Cytokine Levels Using BD Flex Sets

The cytokines from supernatants of activated T cells were also quantified by using a Flex Set 8-plex to measure IL-2, IL-4, IL-5, IL-6, IL-10, TNF, IFN-γ, and IL-17A. The data is presented in Figure 10.

The cytokine data from human PBMCs stimulated with immobilized anti-human CD3 antibody, soluble anti-human CD28 antibody, recombinant human IL-2, and recombinant human IL-4, followed by re-stimulation with PMA and ionomycin clearly shows production of IL-2, IL-4, TNF, and IFN-γ as analyzed by the BD CBA Kit. The IL-2 levels in the samples were too high even at the 1:16 dilutions that were used and therefore were out of range for the IL-2 standard curve. A similar set of cytokines was analyzed using BD Flex Sets along with an

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Figure 10. Analysis of BD Flex Set data in FCAP Array.
Panel A. Standard curve of IL-5.
Panel B. CBA NIR-A vs CBA Red-A dot plot showing eight bead populations with distinct fluorescence intensities.
Panel C. Bar graph showing concentrations of all the cytokines (pg/mL for 1:16 dilution of sample).

additional cytokine, IL-5, which also revealed higher concentrations of IL-2, IL-4, TNF, IFN-γ, and IL-5. Overall, the data presented clearly shows that the stimulation protocol led to secretion of cytokines in the culture supernatants which can be quantified by using BD CBA Kits and Flex Sets.

Conclusions

BD CBA technology is a powerful tool for T-cell research. BD FACSVerse templates provide optimal acquisition settings along with keywords for acquisition of CBA data on the BD FACSVerse system. Further, BD FACSVerse data can be analyzed in FCAP Array v3.0 software which provides automated identification of samples and standards, and calculates the concentrations of cytokines. In this application note, workflows for acquisition of CBA data on the BD FACSVerse system and for analysis of CBA data in FCAP Array v3.0 have been presented using a proof-of-principle protocol for stimulation of human PBMCs. The BD CBA assay platform, combined with the BD FACSVerse flow cytometer, BD FACSuite software, and FCAP Array analysis software, provides an easy and comprehensive solution that creates a powerful multiplexing assay system yielding a variety of benefits compared to conventional ELISA-based platforms.

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6. BD FACSVerse System User's Guide. 23-11463-00 Rev 0.1.
7. FCAP Array™ Software Version 3.0 User's Guide. 23-11472-00 Rev. 01.

Tips and Tricks

- The protocol described for activation of T cells can be used for secretion of IL-2, IL-4, IL-5, IL-6, IL-10, TNF, IFN-γ, and IL-17A.
- The pre-defined tube settings in the CBA templates can be further modified before creation of a CBA assay.
- When using the Loader option to run samples in multi-well plates, we recommend creating the worklist before assigning the samples and standards to the wells in the plate.
- For each sample, we recommend providing a unique ID in the worklist. For replicate samples, the same sample ID should be used. This facilitates the analysis in FCAP Array software which identifies the unique and replicate samples by the BD FACSuite sample ID.
- We recommend testing samples at various dilutions to accurately determine cytokine concentrations based on the standard curve because of variation in the production levels of different cytokines.
- If using frozen serum or plasma samples, we recommend centrifuging the samples after thawing to remove debris as it may interfere with the CBA protocol.
- Performing a final wash in CBA protocol can minimize assay background.
- CBA template experiments and user's guides are available at **bdbiosciences.com/cbasetup**.