Chapter 2

BD™ Cytometric Bead Array (CBA) Multiplex Assays

Introduction

Flow cytometry is a powerful analytical tool that enables the characterization of cells and subcellular organelles as well as particles (eg, plastic beads) on the basis of size and granularity (light scatter characteristics) and a number of different parameters defined by fluorescent probes (including fluorescent antibodies and dyes).[1, 2] Recently, flow cytometry has been applied to the development of multiplex sandwich immunoassays.[3-5] These particle-based, flow cytometric immunoassays are capable of simultaneously identifying the types and measuring the levels of multiple different molecules (aka, antigens, analytes) within small samples of biological fluids. The broad dynamic range of fluorescent detection offered by flow cytometry and the efficient capturing of analytes by suspended particles enables these assays to use fewer sample dilutions and to obtain multiple sample measurements in a short time period. For these reasons, this technology provides an extremely important tool for analyzing the networks of biological response modifiers (BRMs) that are coexpressed by cells that mediate immune and inflammatory responses. BRMs such as cytokines, chemokines, inflammatory mediators (eg, bioactive complement fragments), and their receptors, as well as immunoglobulins, are popular target molecules for study.[3-5] In addition, these assays can be applied to the multiplex analysis of cell signaling molecules that act in complex pathways to orchestrate cellular responses.[6]

The multiplex BD™ Cytometric Bead Array (CBA) Kit employs a series of different particles that are stably labeled with a fluorescent dye whose emission wavelength is read at ~650 nm.[3] Each different group of beads is labeled with a discrete level of fluorescent dye so that it can be distinguished by its mean fluorescence intensity (MFI) upon flow cytometric analysis. In addition, beads within each group are covalently coupled with antibodies that can specifically capture a particular type of molecule present within biological fluids including sera, plasma[5], tears, tissue culture supernatants, or cell lysates. By analogy with the ELISA method (described in ***Chapters 7 and 8***), the antibody-coupled "Capture Beads" serve as the "solid capture phase" for the Cytometric Bead Array. The immobilized, high-affinity antibodies function to specifically capture and localize analytes of interest that may be present in biological fluids.

The captured analyte is then specifically "detected" by the addition of a fluorescent antibody. Fluorescein isothiocyanate-(FITC)(~530nm) and phycoerythrin (PE)(~585nm) coupled detection antibodies (whose wavelengths are distinguishable from the fluorescence signals emitted by the dyed CBA Capture Beads) are often used. By including serial dilutions of a standard analyte solution (eg, a mixture of cytokine protein standards with known concentrations), the CBA supports the development of standard curves (aka, calibration curves) for

each analyte. With multicolor flow cytometric analysis, the levels of analytes (proportional to the bound detection antibody MFI signals) captured by the different bead groups (distinguished by their MFI signals) are measured. The data is analyzed through use of the BD CBA Software to calculate the concentrations of multiple analytes that may be coexpressed within biological fluid samples. Due to the complexity of the BRM and cell signaling networks that underlie immune function, the BD CBA Kit's capacity to simultaneously measure multiple analytes in a single small-volume sample is highly advantageous.

The list of BD Cytometric Bead Array products is growing. Presently, there are several BD CBA Kits for measuring human and mouse cytokines related to Type 1 and Type 2 Immune Responses and Inflammatory Responses. New BD CBA Human Kits for measuring Active Caspase-3 (involved in apoptosis) and Anaphylatoxic Complement Fragments (C3a, C4a, and C5a) are available. A BD CBA Kit that enables the determination of the heavy and light chain isotypes of mouse immunoglobulins is also offered. Other CBA and accessory products include lyophilized CBA standards, BD CBA Software, and the BD Multiwell™ AutoSampler that can be used to increase the throughput and decrease the hands-on time for performing CBA Assays. For more information concerning BD CBA products, please access the BD Biosciences website, **www.bdbiosciences.com/pharmingen/CBA/**

Principle of the Test

Specific descriptions and instructions are provided with each different BD CBA Kit. In general, BD CBA Kits can simultaneously and quantitatively measure multiple analytes (proteins) in a single sample. Each kit's performance has been optimized for analysis of specific analytes in tissue culture supernatants, EDTA-treated plasma and serum samples, or cell lysates. The BD CBA Capture Bead population(s), each with distinct fluorescence intensities (read at ~650 nm/FL3 by BD FACS™ brand flow cytometers), have been coated with capture antibodies specific for various analytes.

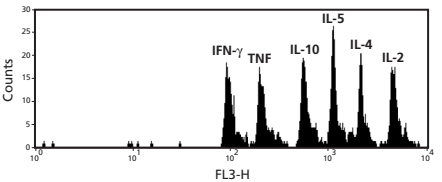

Figure 1. Representative fluorescence (FL3-H) frequency distributions for the Capture Bead populations from the BD CBA Human Th1/Th2 Cytokine Kit.

The BD CBA Capture Beads are mixed with fluorescent (eg, PE-conjugated) detection antibodies and standards, controls, or test samples, to form sandwich complexes (eg, Capture Bead-Ab/analyte/PE-Ab complexes). Following acquisition of sample data using multicolor flow cytometry, the sample results are generated in a graphical and tabular format using the BD CBA Software.

Advantages

The BD CBA Kits provide several advantages when compared with some immunoassay methods. For example, the required sample volume for measuring multiple analytes is smaller than some conventional immunoassays wherein only one analyte can be measured per sample. Due to the BD CBA Kit's capacity to detect six analytes in a single sample, the BD CBA Human Th1/Th2 Cytokine Kit requires approximately one-sixth the sample volume required when compared with a conventional immunoassay. The capacity to use smaller sample volumes is an extremely important feature of the multiplex BD CBA, as precious samples are often available in only limited quantities.[4] The generation of standard curves for multiple analytes is simplified since the analyte standards are often provided as a mixture, thereby requiring no preparation of standard mixtures before making serial dilutions. Moreover, due to the extended dynamic range of BD CBA's when compared with conventional immunoassays, fewer serial dilutions of samples may be required. Altogether, these features can help make BD CBA experiments take less time to perform than individual immunoassays.

Limitations

The BD CBA is not recommended for use with stream-in-air flow cytometers. Fluorescent signal intensities may be reduced with these instruments and adversely affect the assay sensitivity. Stream-in-air instruments include the BD FACStar™ Plus and BD FACSVantage™ (BD Biosciences Immunocytometry Systems, San Jose, CA) flow cytometers.

Reagents Provided

Each BD CBA Kit includes specific Capture Beads, Detection Reagents, Standards, assay buffers, and Flow Cytometer Setup Reagents. All of the reagents required for performing a BD CBA experiment are provided in each BD CBA Kit.

Materials Required but not Provided

In addition to the reagents provided in a BD CBA Kit, the following items are also required:

a. A flow cytometer equipped with a 488 nm laser capable of detecting and distinguishing fluorescence emissions at 576 and 670 nm (eg, BD FACScan™ or BD FACSCalibur™ systems) and BD CellQuest™ Software.

b. 12 × 75 mm sample acquisition tubes for a flow cytometer (eg, BD Falcon™, Cat. No. 352008).

c. BD CBA Software (Cat. No. 550065).

Note: For use with BD CellQuest Software, Microsoft® Excel and a Macintosh or PC-compatible computer are required to utilize the BD CBA Software. See the BD CBA Software User's Guide for details.

d. BD CaliBRITE™ 3 Beads (Cat. No. 340486).

BD CBA Assay Procedures

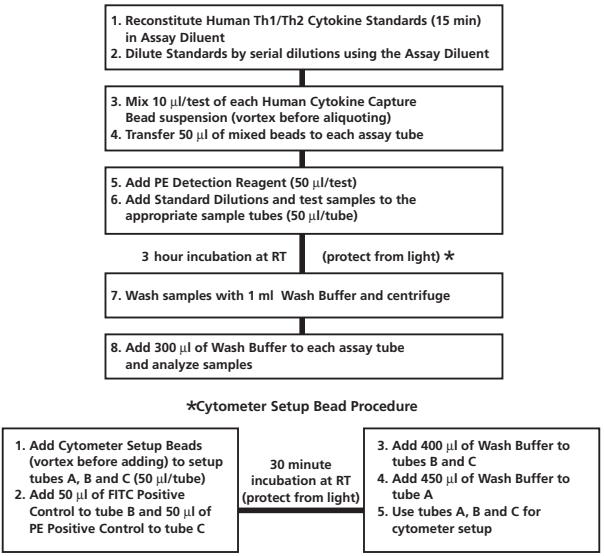

Figure 2. Overview: BD CBA Human Th1/Th2 Cytokine Kit Assay Protocol

Each of the BD CBA Kits are specific for proteins in a variety of matrices and often have differences in their specific protocols. For information on the protocol used by a given BD CBA Kit, please refer to the specific BD CBA Kit Manual that can be downloaded from the BD Biosciences website at:
www.bdbiosciences.com/pharmingen/CBA/

Preparation of BD CBA Assay Standards

Each BD CBA Kit contains standard mixtures in an easy-to-use format. The lyophilized standards (once reconstituted) or the standards provided at 4°C, are serially diluted before mixing with the Capture Beads and the Detection Reagent in a given assay.

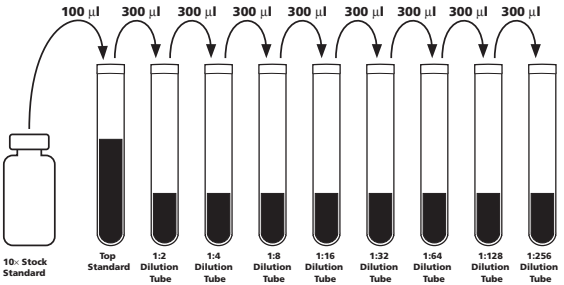

Figure 3. Example standards serial dilutions for the BD CBA Human Th1/Th2 Cytokine Kit.

An example of the approximate concentration (pg/ml) of recombinant protein in each dilution tube in the BD CBA Human Th1/Th2 Cytokine Kit is shown in *Table 1*.

Protein (pg/ml)	Top Standard	1:2 Dilution Tube	1:4 Dilution Tube	1:8 Dilution Tube	1:16 Dilution Tube	1:32 Dilution Tube	1:64 Dilution Tube	1:128 Dilution Tube	1:256 Dilution Tube
Human IL-2	5000	2500	1250	625	312.5	156	80	40	20
Human IL-4	5000	2500	1250	625	312.5	156	80	40	20
Human IL-5	5000	2500	1250	625	312.5	156	80	40	20
Human IL-10	5000	2500	1250	625	312.5	156	80	40	20
Human TNF-α	5000	2500	1250	625	312.5	156	80	40	20
Human IFN-γ	5000	2500	1250	625	312.5	156	80	40	20

Table 1. BD CBA Human Th1/Th2 Cytokine Standard Concentrations after Dilutions.

Cytometer Setup, Data Acquisition, and Analysis

For optimal performance of a BD CBA assay, it is necessary to properly set up the flow cytometer. For this purpose, each BD CBA Kit uses a simple procedure and templates to enable the operator to optimize their instrument setup. The cytometer setup information in this section is for the BD FACScan and BD FACSCalibur flow cytometers. The BD FACSComp™ Software is useful for setting up the flow cytometer. BD CellQuest Software is required for analyzing samples and formatting data for subsequent analysis using the BD CBA Software.

Instrument Setup with BD FACSComp Software and BD CaliBRITE Beads

1. Add 50 µl of Cytometer Setup Beads to three cytometer setup tubes labeled A, B and C.
2. Add 50 µl of FITC Positive Control Detector to tube B.
3. Add 50 µl of PE Positive Control Detector to tube C.
4. Incubate tubes A, B and C for 30 minutes at room temperature and protect from direct exposure to light.
5. Add 450 µl of Wash Buffer to tube A and 400 µl of Wash Buffer to tubes B and C.

Instrument Setup with BD FACSComp Software and BD CaliBRITE Beads

1. Perform instrument start up.
2. Perform flow check.
3. Prepare tubes of BD CaliBRITE Beads and open BD FACSComp Software.
4. Launch BD FACSComp Software
5. Run BD FACSComp Software in Lyse/No Wash mode.

6. Proceed to ***Instrument Setup with the Cytometer Setup Beads***.

   Note: 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. Version 4.2 contains a BD CBA preference setting to automatically save a BD CBA calibration file at the successful completion of any Lyse/No Wash assay. The BD CBA calibration file provides the optimization for FSC, SSC, and threshold settings as described in ***Instrument Setup with the Cytometer Setup Beads***, steps 3 – 5. Optimization of the fluorescence parameter settings is still required (ie, PMT and compensation settings, see ***Instrument Setup with the Cytometer Setup Beads***, ***Step 6***).

Instrument Setup with the Cytometer Setup Beads

1. Launch BD CellQuest Software and open the BD CBA Instrument Setup template.

   Note: The BD CBA Instrument Setup template can be found on the BD CBA Software or FACStation CD for Macintosh computers in the BD CBA folder. Following installation on Macintosh computers using BD CBA Software Version 1.0, the template can be found in the BD Applications/BD CBA folder/Sample Files/Mouse Isotyping Files/Instrument Setup folder. For BD CBA Software Version 1.1 or higher, the template can be found in the BD Applications/BD CBA folder. The template is not installed from the CD on PC-compatible computers. This file and instrument setup templates for two-laser and other flow cytometers may also be downloaded via the internet from: **www.bdbiosciences.com/pharmingen/CBA/downloads.shtml**

2. Set the instrument to Acquisition mode.

   Note: The BD CBA Software will evaluate data in five parameters (FSC, SSC, FL1, FL2 and FL3). Turn off additional detectors.

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 FSC at 650.
6. In setup mode, run Cytometer Setup Beads tube A. Follow the setup instructions in the CBA manual.

   Note: Pause and restart acquisition frequently during the instrument setup procedure in order to reset detected values after settings adjustments. Adjust gate R1 so that the singlet bead population is located in gate R1 (***Figure 4a***).

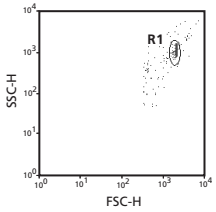

Figure 4a

Adjust gate R1 so that the singlet bead population is located in gate R1 (***Figure 4a***).

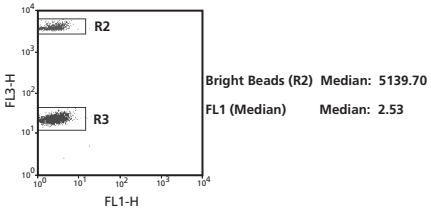

Figure 4b

Adjust the FL3 PMT so that the median of the top FL3 bead population's intensity is approximately 5000 (***Figure 4b***). Adjust gate R3 as necessary so that the dim FL3 bead population is located in gate R3 (***Figure 4b***). Do not adjust the R2 gate.

Adjust the FL1 PMT so that the median of FL1 is approximately 2.0–2.5 (***Figure 4b***).

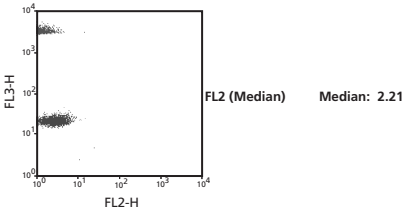

Figure 4c

Adjust the FL2 PMT value so that the median of FL2 is approximately 2.0 – 2.5 (***Figure 4c***).

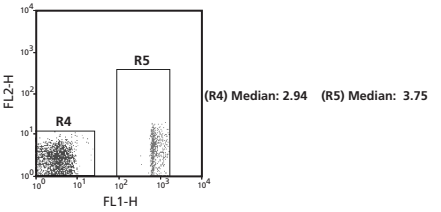

Figure 4d

Run Cytometer Setup Beads tube B to adjust the compensation settings for FL2 – %FL1.

Adjust gate R5 as necessary so that the FL1 bright bead population is located in gate R5 (***Figure 4d***). Using the FL2 – %FL1 control, adjust the median of R5 to equal the median of R4 (***Figure 4d***).

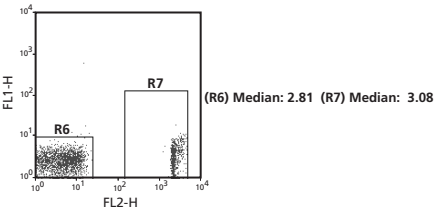

Figure 4e

Run Cytometer Setup Beads tube C to adjust the compensation settings for FL1 – %FL2 and FL3 – %FL2.

Adjust gate R7 so that the FL2 bright bead population is located in gate R7 (***Figure 4e***). Using the FL1 – %FL2 control, adjust the median of R7 to equal the median of R6 (***Figure 4e***).

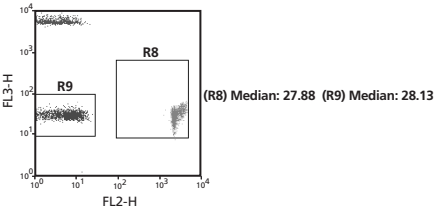

Figure 4f

Adjust gate R9 so that the FL2 bright bead population is located in gate R9 (***Figure 4f***). Using the FL3 – %FL2 control, adjust the median of R9 to equal the median of R8 (***Figure 4f***).

Set the FL2 – %FL3 to 0.1 if necessary. Save and print the optimized instrument settings.

Data Acquisition

1. Open the acquisition template on the BD CBA Software.

   Note: Following installation of the BD CBA Software, the Acquisition template is located in the BD Applications/ BD CBA Folder/Sample Files/Mouse Isotyping Files/Instrument Set Up Folder and is labeled "Isotype Kit Acquire Template". Alternatively, the Acquisition template may be downloaded via the internet from: **www.bdbiosciences.com/pharmingen/CBA/downloads.shtml**

2. Set acquisition mode and retrieve the optimized instrument settings as per the manual.
3. In the Acquisition and Storage window, set the resolution to 1024.
4. Set number of events to be counted as described in the BD CBA kit manual. (This will ensure that the sample file contains approximately 300 events per Capture Bead).
5. Set number of events to be collected to "all events". Saving all events collected will ensure that no true bead events are lost due to incorrect gating.
6. In setup mode, run tube No. 1 and using the FSC vs. SSC dot plot, place the R1 region gate around the singlet bead population (see ***Figure 4a***).
7. Samples are now ready to be read and data acquired.
8. Begin sample acquisition with the flow rate set at HIGH.

   Note: Run the negative control tube (0 pg/ml standards) before any of the recombinant standard tubes. Run the control assay tubes before any unknown test assay tubes.

   To facilitate analysis of data files using the BD CBA 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).

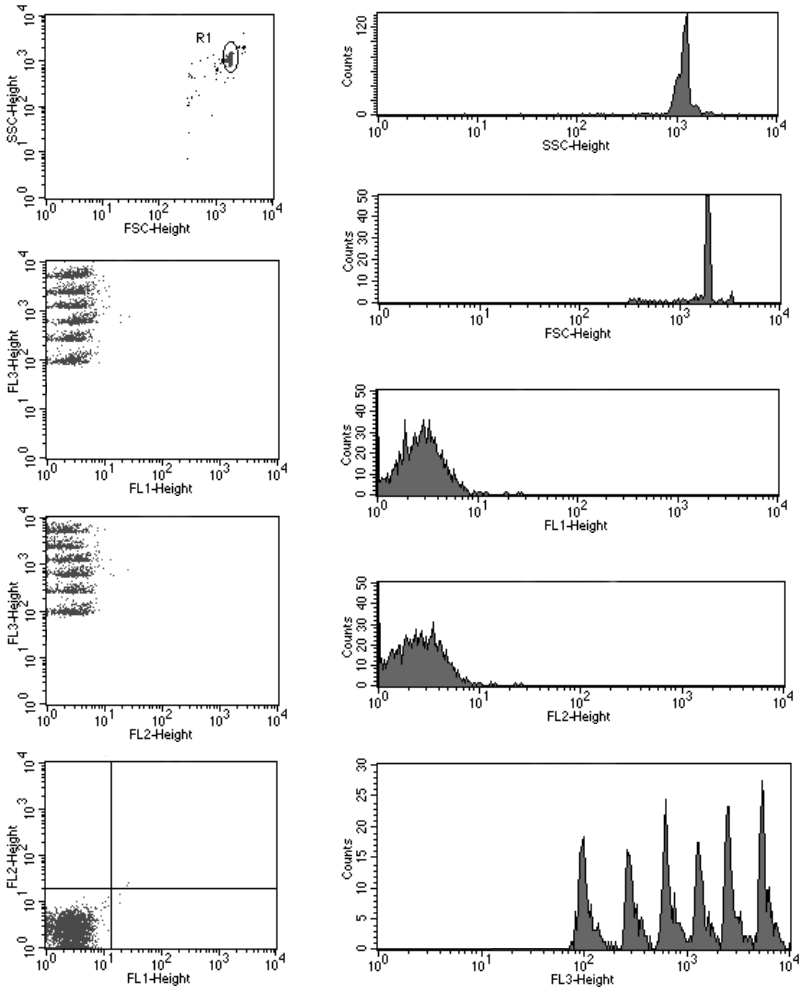

Figure 5. Example Acquisition Template depicting sample data using the BD CBA Human Th1/Th2 Cytokine Kit.

Analysis of Sample Data

The analysis of BD CBA data is optimized when using the BD CBA Software. Install the software according to the instructions in the Software User's Guide. Refer to the manual for each BD CBA Kit for more information on data analysis.

Typical Data

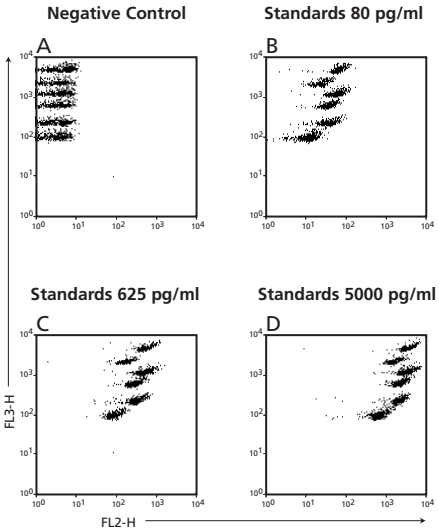

Figure 6. Example BD CellQuest dot plots of various standard dilutions analyzed in the BD CBA Human Th1/Th2 Cytokine Kit.

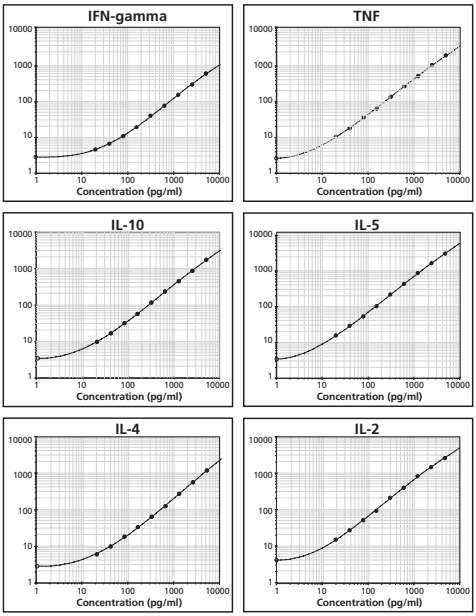

Figure 7. Example standard curves generated using the BD CBA Human Th1/Th2 Cytokine Kit and plotted with the BD CBA Software.

Results

BD Cytometric Bead Array Analysis

8/24/00

					IFN-gamma			TNF		
	Filename	SampleID	Acq Date	Dilut Factor	FL2 MFI	Tube pg/ml	Sample pg/ml	FL2 MFI	Tube pg/ml	Sample pg/ml
1	081500Katy.017	110 supe,8/9/00,neat	15-Aug-00	1	716.9	>5000		3337.6	>5000	
2	081500Katy.018	110 supe,8/9/00,neat	15-Aug-00	1	736.5	>5000		3278.1	>5000	
3	081500Katy.019	110 supe,8/9/00,1/4	15-Aug-00	4	1263.5	>5000		1065.0	3200.3	12801.4
4	081500Katy.020	110 supe,8/9/00,1/4	15-Aug-00	4	1263.5	>5000		1263.5	3863.6	15454.4
5	081500Katy.021	110 supe,8/9/00,1/16	15-Aug-00	16	813.1	>5000		250.3	687.4	10998.1
6	081500Katy.022	110 supe,8/9/00,1/16	15-Aug-00	16	881.7	>5000		324.9	901.5	14424.5
7	081500Katy.023	110 supe,8/9/00,1/64	15-Aug-00	64	271.4	4880.9	312380.6	69.2	182.8	11697.6
8	081500Katy.024	110 supe,8/9/00,1/64	15-Aug-00	64	283.9	5082.7	325295.8	73.7	195.0	12482.7
9	081500Katy.025	110 supe,8/9/00,1/256	15-Aug-00	256	66.1	1442.1	369173.8	18.8	45.9	11748.2
10	081500Katy.026	110 supe,8/9/00,1/256	15-Aug-00	256	65.5	1431.1	366356.1	16.7	40.3	10316.0
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Figure 8. Example sample data analysis using the BD CBA Software.

BD CBA Assay Performance

For more information concerning performance characteristics (eg, sensitivity, spike recovery, dilution linearity, specificity, and intra- and inter-assay precision), please consult the manual for each specific BD CBA Kit. All current BD CBA product manuals are available on the BD Biosciences website at: **www.bdbiosciences.com/pharmingen/CBA/**

Summary

In summary, the BD Cytometric Bead Arrays represent exciting new technology for analyzing the expression of multiple analytes (eg, cytokines, chemokines, inflammatory mediators, immunoglobulins, and cell signaling molecules) that are often found present together in complex mixtures within biological fluids. For more information concerning BD CBA products, please read the references cited below and access the BD Biosciences website, **www.bdbiosciences.com/pharmingen/CBA/**

References

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Related BD Biosciences Literature

Bowman, B., H. Sepulveda, F.-J. Luan, J. Wilson, and J. A. Ember. 2002. Human anaphylatoxin BD CBA. *BD Biosciences HotLines* 7(1):9.

Grantham-Wright, D., and J. Wilson. 2001. New from the Cytometric Bead Array Program at BD Biosciences Pharmingen. *BD Biosciences HotLines* 6(2):4.

Sepulveda, H., H. Baumhover, D. Mochizuki, J. Wilson, and D. Ernst. 2001. Standardization of the Human Th1/Th2 Cytometric Bead Array (CBA) Cytokine Standards to International Standards. *BD Biosciences HotLines* 6(3):8.

Garrett, D. 2001. CBA Software Flexibility and advantages. *BD Biosciences HotLines* 6(1):8.

Ward, T., and D. Grantham. 2000. The BD Cytometric Bead Array System. *BD Biosciences HotLines* 5(3):4.

The BD Cytometric Bead Array System. BD Biosciences Brochure.

BD Cytometric Bead Array Product List

Description	Contains	Apps	Format	Size	Cat. No.
Human					
Anaphylatoxin Kit	C3a, C4a, C5a	FCM	Kit	50 tests	552363
Chemokine Kit	IL-8, RANTES, MIG, MCP-1, IP-10	FCM	Kit	50 tests	552990
Inflammation Kit	IL-8, IL-1β, IL-6, IL-10, TNF, IL-12p70	FCM	Kit	50 tests	551811
Inflammation Standards	IL-8, IL-1β, IL-6, IL-10, TNF, IL-12p70	FCM	Lyophilized	1 vial	552932
Th1/Th2 Cytokine Kit	IL-2, IL-4, IL-5, IL-10, TNF, IFN-γ	FCM	Kit	50 tests	550749
Th1/Th2 Cytokine Kit II	IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ	FCM	Kit	50 tests	551809
Th1/Th2 Cytokine Standards	IL-2, IL-4, IL-5, IL-6, IL-10, TNF, IFN-γ	FCM	Lyophilized	1 vial	551810
Apoptosis Kit	Bcl-2, cleaved PARP Active Caspase-3	FCM	Kit	50 tests	inquire
Active Caspase-3	Caspase-3	FCM	Kit	100 tests	552124
Mouse					
Immunoglobulin Isotyping Kit	Heavy and light chain isotypes of mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, IgM, and IgE	FCM	Kit	1 kit	550026
Inflammation Kit	IL-6, IL-10, MCP-1 IFN-γ, TNF, IL-12p70	FCM	Kit	50 tests	552364
Th1/Th2 Cytokine Kit	IL-2, IL-4, IL-5, TNF, IFN-γ	FCM	Kit	50 tests	551287
Th1/Th2 Cytokine Standards	IL-2, IL-4, IL-5, TNF, IFN-γ	FCM	Lyophilized	1 vial	552967
Other					
Phosphorylated STAT1 Kit	Phosphorylated STAT1	FCM	Kit	100 tests	557740
BD CBA Software	Mac and PC compatible CDROM and User's Guide	FCM		1 CD	550065