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Bead-Based Multianalyte Flow Immunoassays

The Cytometric Bead Array System

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Summary

Analytical cytometry has significant potential beyond cellular analysis. The inherent capability of flow cytometers to efficiently discriminate between uniformly sized particles based on their intrinsic properties provides the foundation for multiplex bead assays. The technology can be exploited in designing immunoassays, Western blot-like antibody assays, and nucleic acid hybridization assays. This chapter focuses on immunoassay applications. The multiplex bead assays have recently evolved as a new and increasingly popular area for flow cytometry, becoming a good alternative to enzyme-linked immunosorbent assay for efficient evaluation of panels of analytes. This chapter provides detailed information about two bead platforms, the BD™ Cytometric Bead Array kits and the BD Cytometric Bead Array Flex Set Assays.

Key Words: CBA; multiplex bead immunoassays; preconfigured kits; Flex-Set assays; soluble proteins; cell signaling proteins; cell lysates.

1. Introduction

Bead-based, flow cytometric immunoassays have the ability to simultaneously and quantitatively measure multiple antigens or antibodies in a small volume of biological fluids. The flow cytometers support a broad dynamic assay range for the multiplex assays. The technology may be utilized to analyze the networks of mediators expressed by cells during immune and inflammatory responses. Cytokines ***(1)***, chemokines ***(2)***, inflammatory mediators and their receptors, as well as immunoglobulins ***(3)***, are frequently described as target molecules for multiplex assays. In addition, the bead assays can be applied to the simultaneous analysis of cell signaling molecules ***(4)*** and follow various activation pathways.

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The use of microspheres of different size or color is at the basis of constructing multiplexed immunoassays. Several analytes can be assayed in one tube using very small sample volumes. Three basic concepts were developed to establish multianalyte assays. Fulwyler ***(5)*** and McHugh ***(6)*** pioneered the flow multiplex area using beads of different sizes as carriers for antigens or antibodies. The beads carrying different analytes are differentiated by their different scatter characteristics. Binding of fluorescent detectors to the beads generate the immunoassay signal.

Beads of the same size may be identified and differentiated by one type of fluorescence, whereas the signal is generated by conjugates carrying a second type of fluorescent signal ***(7)***. This concept is useful to create low-complexity bead sets ***(8)***.

Combining two or more fluorescent indexing colors in individual beads further extended the usefulness of multiplex flow assays. The individual bead populations contain unique ratios of the incorporated dyes, and their differing fluorescent signature is used to identify a series of beads carrying different specificities. Bead indexing is achieved in multidimensional fluorescent space with the capability to reach up to 100 individual bead addresses in two dimensions ***(9)***. The immunoassay signal is generated by antibodies coupled to a fluorescent dye, which is not interfering with the bead indexing. New instruments were designed to exploit the capabilities of multiplex bead assays without requiring the complex expertise of flow cytometry. The LabMAP 100 by Luminex and the BD FACSArray Bioanalyzer (**Fig. 1**) by BD Biosciences both support the utilization of the familiar microplate format. Both of these instruments have two lasers, a red laser that excites the two dyes in the dual color beads, and a green laser that is used for exciting the bead-bound conjugates. The signal generator is most often phycoerythrin (PE), thus the green laser excitation maximizes signal-to-noise ratios.

Multiplex assays are well suited to demonstrate a pattern of antibody responses against infectious agents, thus providing a bead assay analog to the Western blot method. McHugh ***(10)*** presented a prototype Hepatitis C virus antibody assay for potential use in the blood bank. Faucher ***(11)*** has demonstrated the capability of the multiplex assays to detect HIV-1 antibodies from fresh plasma and from dried bloodspot specimens. Khan ***(12)*** utilized the multiplex format to construct a serological assay, which detected 10 highly prevalent mouse infectious pathogens in a single reaction.

Two types of cytometric bead array (CBA) assays were developed at BD BioSciences. The BD CBA kits contain all the necessary components to perform the assay. The BD CBA Flex Set assays, on the other hand, provide all the necessary components and protocols to create customized multiplex panels, allowing a mix-and-match strategy.

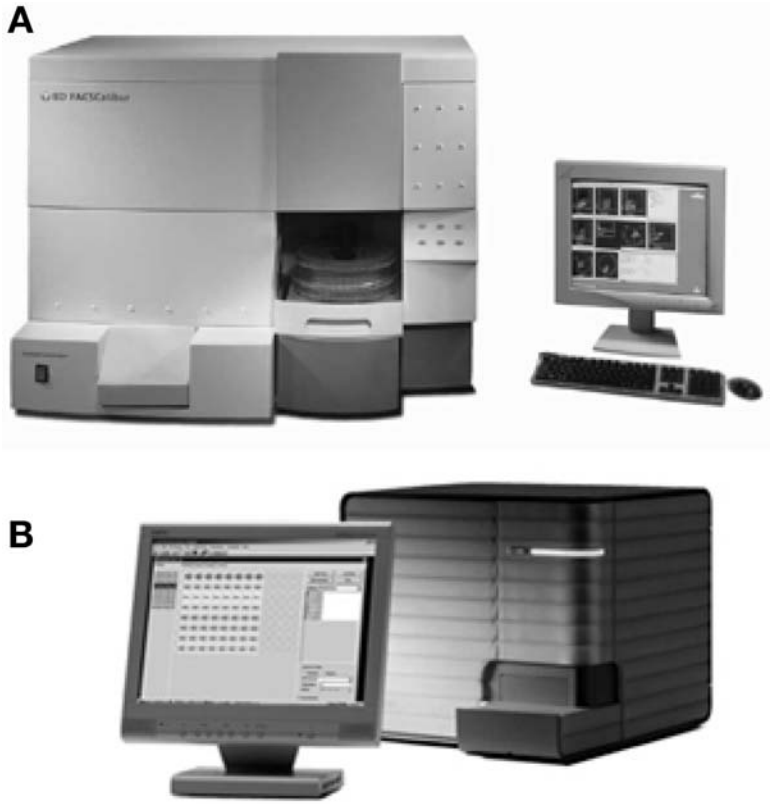


Fig. 1. BD Flow cytometers most often used with the cytometric bead array (CBA) assays. **(A)** BD FACSCalibur™ Flow Cytometer. The dual laser instrument is compatible with single color and dual color indexed CBA beads and assays constructed with these beads. **(B)** BD FACSArray™ bioanalyzer is a microplate-based instrument optimized for bead assays. The instrument utilizes a 635-nm red laser to index the dual color beads and a 532-nm green laser for exciting the signal generator phycoerythrin (PE) conjugate. Both single color and dual color indexed beads are compatible with the platform.

The CBA kits are utilizing beads, which are dyed with a single red fluorescent dye. 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. Beads within each group are covalently coupled with antibodies that can specifically capture a particular type of molecule present within biological fluids including serum, plasma, tissue culture supernatants, or cell lysates. The capture beads are mixed with PE-conjugated detection antibodies and standards, controls, or test samples to form sandwich complexes. Following acquisition of sample data using multicolor flow cytometry, the

Table 1
List of Available BD™ CBA Kits

Human CBA kits	
Kit name	Specificities in kit
Allergy/asthma mediator kit – I	IL-3, IL-4, IL-5, IL-7, IL-10, GM-CSF
Allergy/asthma mediator kit – II	IL-3, IL-4, GM-CSF, G-CSF, eotaxin
Anaphylatoxin	C4a, C3a, C5a
Apoptosis kit	Cleaved PARP, Bcl-2, active caspase-3
Chemokine kit – I	IL-8, RANTES, MIG, MCP-1, IP-10
Inflammation kit	IL-8, IL-1β, IL-6, IL-10, TNF-α, IL-12p70
Th1/Th2 cytokine kit – I	IL-2, IL-4, IL-5, IL-10, TNF-α, IFN-γ
Th1/Th2 cytokine kit – II	IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ
Nonhuman primate CBA kits	
Nonhuman primate Th1/Th2 kit	IL-2, IL-4, IL-5, IL-6, TNF-α, IFN-γ
Mouse CBA kits	
Immunoglobulin isotyping kit	IgG1, IgG2a, IgG2b ,IgG3 , IgA, IgM, IgE
Inflammation kit	IL-6, IL-10, MCP-1, IFN-γ, TNF-α, IL-12p70
Th1/Th2 cytokine kit	IL-2, IL-4, IL-5, TNF, IFN-γ

standard curves are compiled in graphic format and sample results are tabulated by the BD CBA software.

The assays are compatible with most of the BD flow cytometers, including FACScan, FACSCalibur, FACSVantage, FACSAria, FACSCanto, and FACSArray platforms. The performance characteristics of the multiplex assays, including sensitivity, spike recovery, dilution linearity, specificity, and intra- and interassay precision were determined for each kit.

The list of available CBA kits is summarized in **Table 1**. The immunoassay analysis is performed by a stand alone CBA software, which is compatible with both Apple Macintosh and PC computers. **Figure 2** shows six standard curves constructed with data using the Th1/Th2 CBA panel.

The CBA kits are often utilized to profile immunological processes. Tear samples from nonallergic and allergic donors were tested and reduced levels of interleukin (IL)-10 was found in allergic donors as compared to nonallergics ***(13)***. This application was further developed by Sonoda ***(14)***. Inflammatory cytokine CBA panel was used to monitor IL-6, IL-8, and IL-10 levels in pediatric patients who underwent cardiopulmonary bypass procedure ***(15)***. A characteristic time-course of these cytokines was detected, with significant increase during the intraoperative phase and fast decrease in the postoperative phase.

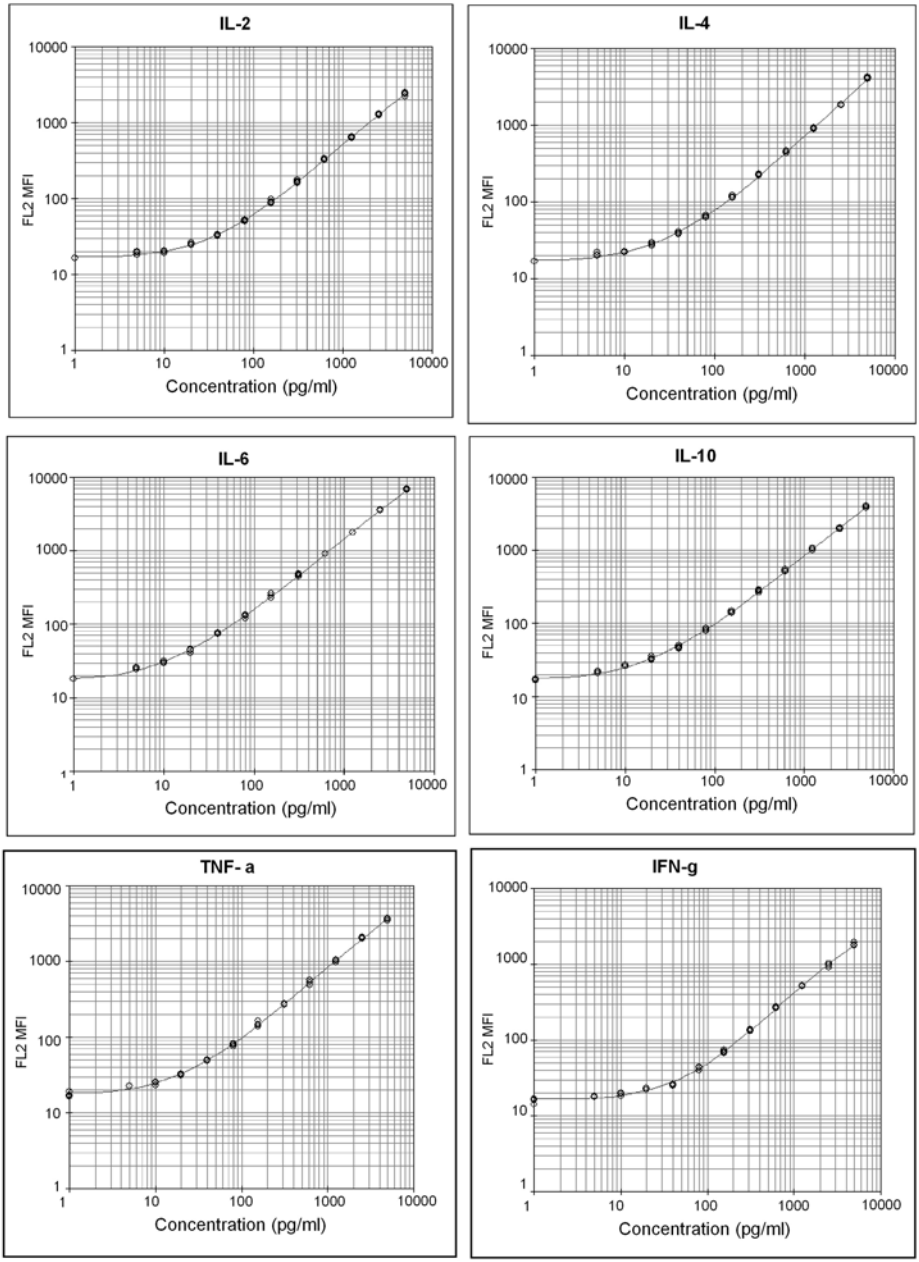


Fig. 2. Standard curves for the BD™ CBA Human Th1/Th2 panel (interleukin [IL]-2, IL-4, IL-6, IL-10, tumor necrosis factor-α, and interferon-γ), analyzed using BD CBA software.

Hodges evaluated plasma samples from neonates with confirmed bacterial infection using the inflammatory cytokine panel ***(16)***. IL-6, IL-10, and IL-12 showed significant increase in *in utero* infected cases, whereas infection acquired after birth did not result in increased cytokine expression. Lipopolysaccharide induced cytokine and chemokine expression was evaluated in human carotid lesions using inflammatory cytokine, Th1/Th2 cytokine, and chemokine panels ***(17)***. The chemokine panel was also useful in analyzing differences between uncomplicated influenza A and B cases and H5N1 influenza A infection ***(18)***.

The kits were also evaluated in combination with cellular assays. Orfao reported simultaneous detection of secreted and cell-bound Th1/Th2 cytokines ***(19)*** using the CBA system.

Specificities and number of analytes are fixed in the CBA kits. Flexibility in combining analytes was achieved with BD CBA Flex Set assays, which are based on dual-color dyed beads (**Fig. 3B**).

The Flex Set system provides an open and configurable menu of bead-based reagents designed to create multiplex assays to specified requirements. Antibody conjugation chemistry, pair optimization strategies, and direct PE-detection reagents assure consistent assay performance in complex biological samples. Each antibody pair is evaluated for dynamic range, sensitivity, and parallel titration to native biological samples. The assay diluent and wash buffers are formulated to reduce potential interferences of serum and plasma samples. Direct PE conjugates are used as detection reagents, this minimizes the risk of increased background caused by endogenous biotin. The Flex Sets are compatible with serum, plasma, tissue culture supernatant, or cell lysate samples. A list of the available specificities and their bead location is summarized in **Table 2**. The standards are provided as unit-dose pellets, which can be easily combined with any number of additional pelletized standards. Common assay components, such as setup particles, buffers, and diluents are combined in a master buffer kit. The assays may be run either in tubes or in microplate format.

The Flex Set reagents require the use of dual-laser flow cytometers capable of detecting and distinguishing fluorescence emissions at 576, 670, and >680 nm. The Flex Set assays are compatible with the dual laser FACSCalibur, LSRII, FACSAria, FACSVantage, FACSCanto instruments, and the FACSArray Bioanalyzer.

Data analysis of the acquired FCS 2.0 data files is performed using FCAP Array software, which automatically clusters dual color CBA beads. It is a template-based system, which allows the design of customized Flex Set assay at the computer workstation. **Fig. 4** shows two standard curves, constructed for IL-6 and IL-8 by using the FCAP Array software. A 27-plex Flex Set assay, consisting of cytokines, chemokines, and other biological modifiers is demonstrated on **Fig. 5**.

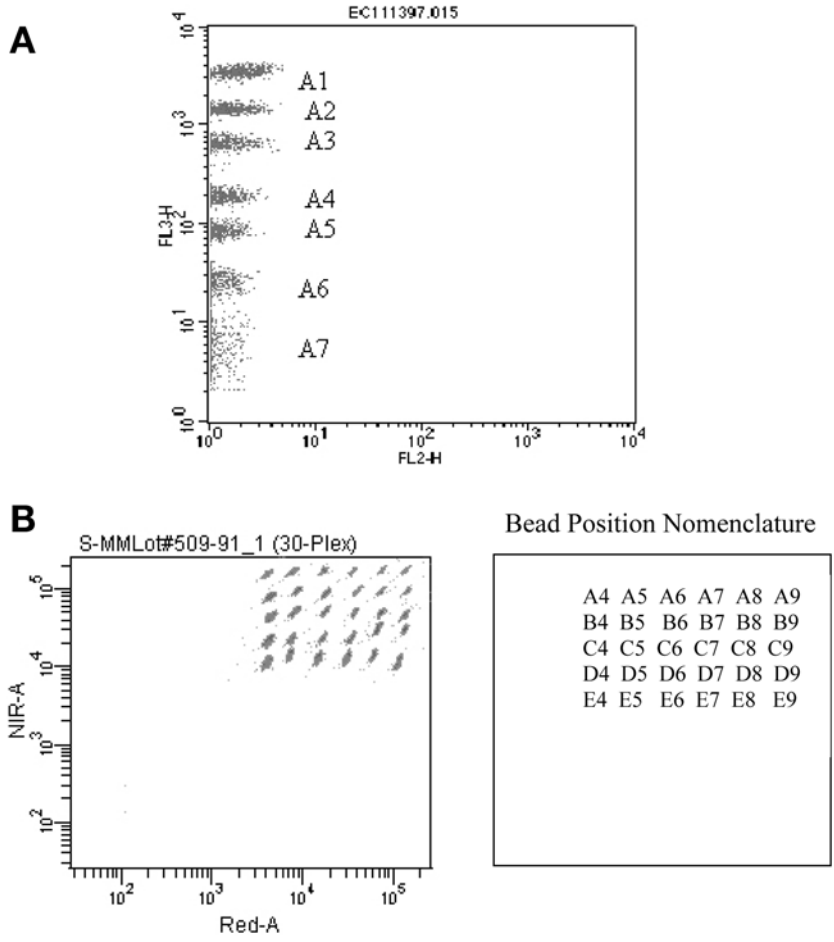


Fig. 3. Bead sets utilized in cytometric bead array (CBA) assays. **(A)** Single color fluorescent beads of graded red fluorescence. These uniform sized beads are discernable by their varying FL3 fluorescence. The single color beads are used in the BD™ CBA assay kits. **(B)** Dual color fluorescent beads. The 30 uniform sized beads are excited by the red laser and discernable in two dimensions by their individual red and near infrared fluorescence. The beads are utilized in the BD CBA Flex Set assays.

Fig. 6 shows the bead map of a 9-plex Flex Set assay which was configured to quantitatively measure T-cell activation in cell lysates. **Fig. 7** demonstrates the kinetics of Jurkat cell activation with CD3/CD28 treatment. Flex set assays were used to evaluate IgM signaling enhancement through ZAP 70 in chronic lymphoid leukemia ***(20)***.

In this chapter, we summarize the specifics of both the fixed panels and the Flex Set CBA assays. We are focusing on the assay methodology, for detailed

Table 2
List of Available BD™ CBA Flex Set Assays

Human soluble protein flex sets			
Analyte	Bead position	Analyte	Bead position
Angiogenin	C4	IL-8	A9
Eotaxin	C7	IL-9	B6
Fas ligand	C6	IL-10	B7
Basic FGF	C5	IL-12p70	E5
G-CSF	C8	IP-10	B5
GM-CSF	C9	LT-a	D7
IFN-γ	E7	MCP-1	D8
IL-1β	B4	MIG	E8
IL-2	A4	MIP-1a	B9
IL-3	D5	MIP-1b	E4
IL-4	A5	RANTES	D4
IL-5	A6	TNFα	D9
IL-6	A7	VEGF	B8
IL-7	A8		
Mouse soluble protein flex sets			
GM-CSF	B9	IL-9	B5
IFN-γ	A4	IL-10	C4
IL-2	A5	IL-12p70	D7
IL-3	A8	IL-13	B8
IL-4	A7	KC	A9
IL-5	A6	MCP-1	B7
IL-6	B4	TNF-α	C8
Rat soluble protein flex sets			
IFN-γ	A6	IL10	A9
IL-4	B9	TNF-α	C8
IL-6	A9		
Phosphorylation site-specific flex sets			
Btk (Y551)[a]	D5	PLCg (Y783)	B7
ERK1/2(T202/Y204)	C4	RSK (T573)	D7
Itk (Y511)	C6	Stat1 (Y701)	C5
JNK1/2 (T183/Y185)	B5	Syk (Y352)	B9
eNOS (S1177)	C7	ZAP-70 (Y319)	B8
p38/MAPKinase (T180/Y182)	B6		

(*Continued*)

Table 2 (*Continued*)

Total signaling protein flex sets			
Stat1	D4	ZAP-70	B8
Syk	B9		

[a]The assays are specific for the phosphorylation sites displayed in brackets for each cell signaling molecule.

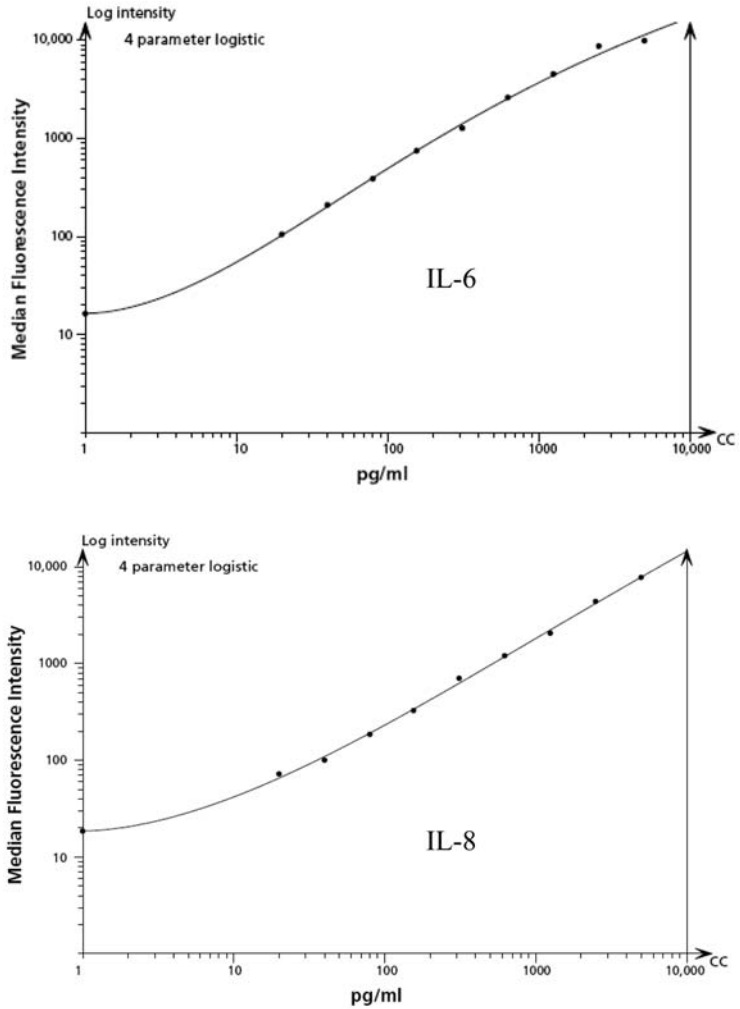
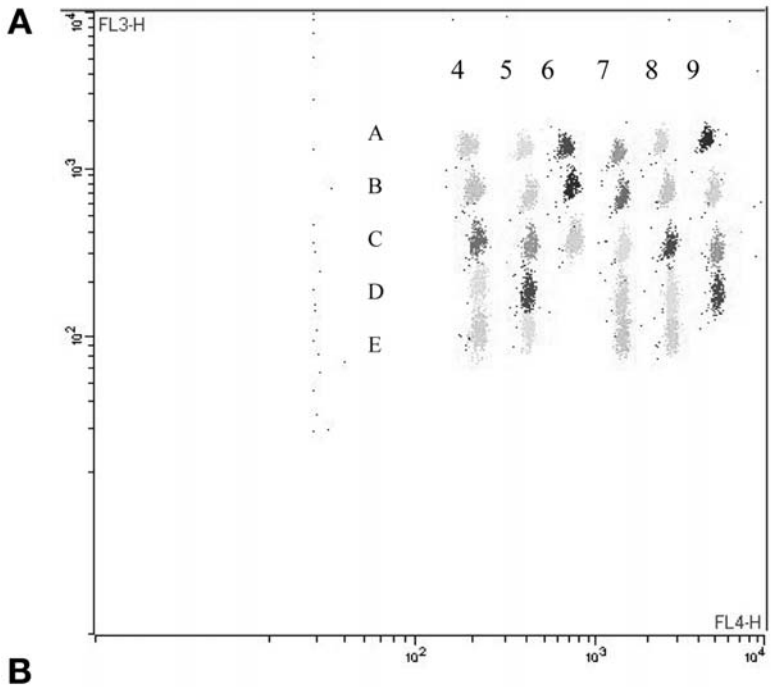


Fig. 4. CBA Flex Set standard curves for human interleukin (IL)-6 and IL-8. Data acquired on a BD FACSArray™ bioanalyzer and analyzed using the FCAP Array™ software.



Analyte	Position	Analyte	Position	Analyte	Position	Analyte	Position
IL-2	A4	IL-9	B6	G-CSF	C8	IL-12p70	E5
IL-4	A5	IL-10	B7	GM-CSF	C9	IFNg	E7
IL-5	A6	VEGF	B8	RANTES	D4	MIG	E8
IL-6	A7	MIP-1a	B9	IL-3	D5		
IL-7	A8	Angiogenin	C4	LT-a	D7		
IL-8	A9	b-FGF	C5	MCP-1	D8		
IL_1b	B4	FasL	C6	TNF	D9		
IP-10	B5	Eotaxin	C7	MIP-1b	E4		

Fig. 5. A 27-plex Flex Set on a dual laser BD FACSCalibur™ instrument. **(A)** Dot plot representation of the 27-plex Flex Set. **(B)** List of the human specificities coupled to the different beads. Each unique bead position is defined as an alphanumeric address.

information on data handling using the BD CBA analysis software and FCAP Array software please refer to their respective user guides. Both of these documents are accessible at www.bdbiosciences.com. Additional technical information about FCAP Array software can be found at the Soft Flow website (www.softflow.com).

2. Materials

2.1. Th1/Th2 Human Cytokine Panel

This panel serves as an example of the ready-to-use CBA kits, which contain all the necessary assay components in a single package. Six bead populations

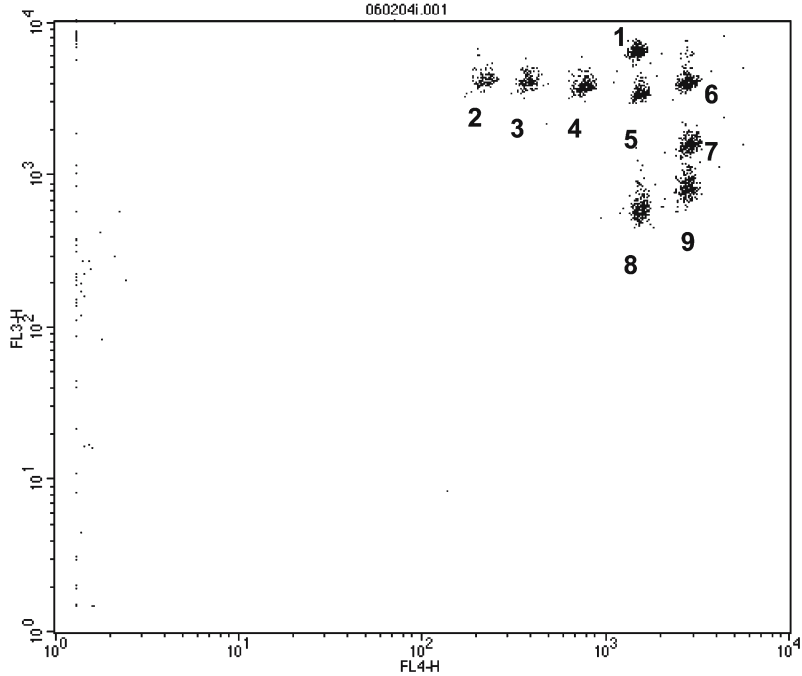


Fig. 6. A 9-plex Flex Set for testing T-cell activation. Key to the bead specificities: (1) Itk, (2) ERK, (3) JNK, (4) P38, (5) PLCγ, (6) ZAP70, (7) LAT, (8) c-Jun, (9) RSK.

with distinct fluorescence intensities have been coated with capture antibodies specific for IL-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ. The six individual bead populations are mixed together during the assay preparation. **Fig. 8** shows the FL3 histogram of the combined capture beads. The cytokine capture beads are combined with the PE-conjugated detection antibodies and then incubated with recombinant standards or test samples to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results are generated in graphical and tabular format using the BD CBA analysis software. The kit provides sufficient reagents for the quantitative analysis of 50 test samples and two standard curve sets.

2.1.1. Human Cytokine Capture Beads

There are 0.8 mL of each specific capture beads with discrete fluorescence intensity characteristics are supplied with the kit. The brightest bead in the kit is designated A1, the dimmest bead is A6. The beads are carrying the following specificities: A1= human IL-2, A2 = human IL-4, A3 = human IL-6, A4 = human IL-10, A5 = human TNF-α, A6 = human IFN-γ. The mixed capture bead

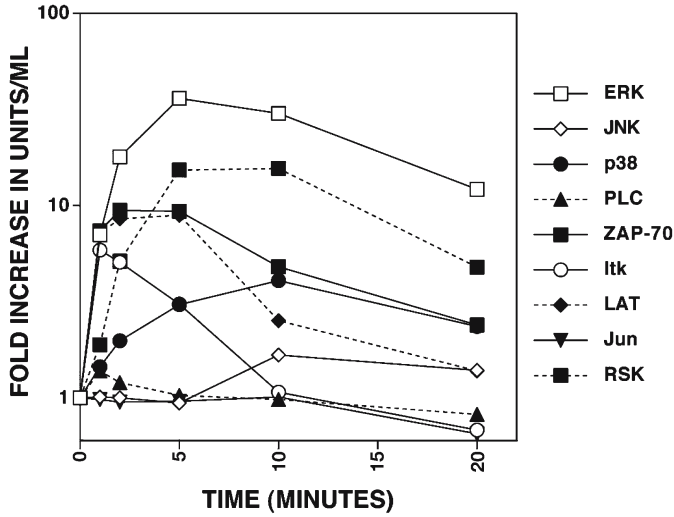


Fig. 7. Kinetics of T-cell activation using anti-CD3/CD28. Jurkat cells were activated with anti-CD3 and anti-CD28 for different lengths of time and the cells were lysed. A 9-plex BD CBA assay using 10 µg of lysate was run measuring phosphorylated ERK, JNK, p38, PLCγ, ZAP-70, Itk, LAT, c-Jun, and RSK. Using standard curves, concentration (U/mL) for each specificity was determined and the fold increase in activity was plotted.

reagent is formulated to support a 50-µL/test volume. The beads need to be stored at 4°C, and cannot be frozen.

2.1.2. PE-detection Reagent

Four milliliters of the mixed detector reagent is provided, containing PE-labeled monoclonal antibodies against human IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ. The combined detector reagent is formulated for use at 50 µL/test.

2.1.3. Cytokine Standards

Two vials of freeze-dried mixed standards are supplied with the kit, each vial containing a mixture of human recombinant IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ. Each vial should be reconstituted in 0.2 mL of assay diluent to prepare a 10X bulk standard. The reconstituted 10X bulk standard contains 50 ng/mL of each recombinant human IL-2, IL-4, IL-6, IL-10, TNF-α, and IFN-γ protein.

2.1.4. Instrument Setup Beads and Controls

One and a half-milliliter setup bead and 0.5 mL of each of a PE- and FITC-labeled controls are included in the kit. The PE control is a PE-conjugated antibody specific for the antigen coated on the setup bead, and formulated for

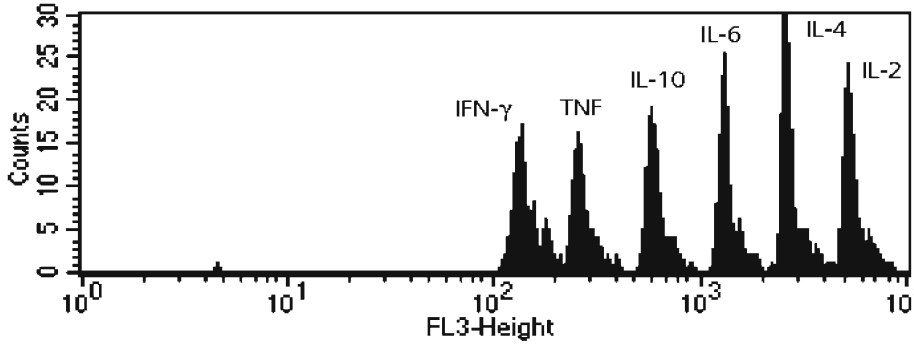


Fig. 8. FL3 histogram of the Th1/Th2 CBA kit beads. The individual bead peaks are labeled with the corresponding specificity.

use at 50 µL/test. The fluorescein (FITC) control is a FITC-conjugated antibody specific for the antigen coated on the setup bead, and formulated for use at 50 µL/test. The controls are used with the instrument setup bead to set the initial instrument compensation.

2.1.5. Buffers

One hundred-thirty milliliters wash buffer and 30 mL assay diluent is supplied in the kit. The wash buffer is phosphate-buffered saline (PBS) with protein and detergent additives. It is used for wash steps and to resuspend the washed beads for analysis. The assay diluent is a buffered protein solution used to reconstitute and dilute the human Th1/Th2 cytokine standards and to dilute test samples.

2.1.6. Instrumentation, Equipment, and Software Requirements

To run the CBA assay a flow cytometer equipped with a 488-nm laser capable of detecting and distinguishing fluorescence emissions at 576 and 670 nm (e.g., BD FACScan™ or BD FACSCalibur™ instruments) and BD CellQuest™ Software is required. Data analysis requires the BD CBA Software. Regular 12 × 75-mm sample acquisition tubes, such as BD Falcon™ tubes are used for the assay preparation and data acquisition.

2.2. Soluble Protein Flex Set Assays

The Soluble Protein Flex Set assay system allows combination of the available single bead assays (**Table 2**) to create multiplex panels, as required by the experimenter's needs. These assays are supporting human, mouse, and rat multiplex panels. Each individual capture bead population has a distinct and unique near-infra red and red fluorescence intensity signature. Each bead species are

covalently coupled with a capture antibody specific for a given soluble protein. Each bead population is resolvable from the other bead species in the multiplex assay by their unique signature in the FL3 and FL4 channels of a FACSCalibur flow cytometer or in the near-infra red and red channels of a FACSArray Bioanalyzer. Each bead population is given an α-numeric position designation indicating its position relative to other beads in the BD CBA Flex Set system (**Table 2**, **Fig. 3B**). Beads with different positions can be combined in assays to create a multiplex assay. In a Flex Set assay the capture bead, PE-conjugated detection reagent, and standard or test samples are incubated together to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results are generated in a graphical and tabular format using the FCAP Array software.

2.2.1. Bead Reagents

1. Flex Set beads: each capture bead species supplied as bead suspensions in buffered saline solution containing fetal bovine serum and 0.09% sodium azide.
2. Instrument setup beads: setup beads defining the four corners of the Flex Set bead clusters (A1, A8, F1, F9 positions) are supplied as single-bead suspensions and are used to adjust the optimal instrument setup.
3. PE instrument setup bead: suspension of F1 beads, used for generating a PE positive bead population for instrument setup. The beads are covalently coupled with an anti-immunoglobulin antibody.

2.2.2. Antibody Reagents

1. Flex Set detectors: each analyte is defined by both the specific capture bead and the complementary PE-conjugated detector antibody reagent. The corresponding bead and detector specificities, together with lyophilized standards are supplied for each analyte in a single package. Each antibody conjugate is supplied in buffered saline solution containing bovine serum albumin (BSA) and 0.09% sodium azide. For the list of available soluble protein analytes to construct multiplex assays, *see* **Table 2**.
2. PE-positive control detector: a single vial of PE-conjugated antibody, formulated for use at 50 µL/test. This reagent is used with the PE instrument setup bead F1 to set instrument compensation settings. Store at 4°C. Do not freeze.

2.2.3. Flex Set Standards

For each assay, two vials of lyophilized standards are provided. The standards are lyophilized from an aqueous buffered protein solution containing BSA and Proclin™ 150. Each vial is reconstituted with 0.2 mL assay diluent.

2.2.4. Buffers and Diluents

1. Wash buffer: 1X PBS solution, containing protein and detergent, used for wash steps and to resuspend beads for analysis. Store at 4°C.

2. Assay diluent: 1X buffered solution used to dilute the BD™ CBA Human Soluble Protein Flex Set Standards and to dilute test samples. Store at 4°C.
3. Capture bead diluent for serum/plasma samples: 1X PBS solution containing protein used to resuspend capture beads prior to testing serum or plasma samples. Store at 4°C.
4. Capture bead diluent for cell culture supernatant samples: 1X PBS solution containing protein used to dilute capture beads prior to testing cell culture samples. Store at 4°C.
5. Detection reagent diluent: 1X PBS solution containing protein used to dilute the detection reagents. Store at 4°C.

2.2.5. Instrumentation, Equipment, and Software

A flow cytometer equipped with a 488- or 532-nm laser and a 635-nm laser capable of detecting and distinguishing fluorescence emission at 576 and 670 nm (off the 488-nm laser) and 660 nm (off the 635-nm laser) such as a FACSCalibur or 576 nm (off the 532-nm laser) and 660 and >680 nm (off the 635-nm laser) such as a FACSArray bioanalyzer is needed to perform CBA Flex Set bead assays. For assays run on the FACSCalibur 12 × 75-mm sample acquisition tubes are needed. FACSArray tests are run on microtiter plates, such as Millipore MultiScreen® BV 1.2-µm clear nonsterile filter plates. Plate washing is performed on a Millipore MultiScreen vacuum manifold, the plates are mixed on a digital microplate stirrer. Data acquisition and analysis requires availability of CellQuest, FACSComp, and FCAP Array software packages.

2.3. Cell Signaling Flex Set Assays

The cell signaling Flex Set assays are two-site sandwich multiplex immunoassays, which are available as total protein or phophorylated protein assays (**Table 2**). The assays are using cell lysates as samples and provide quantitative results based on standard curves constructed with recombinant protein standards. The sample data are analyzed using the FCAP Array software and the results are expressed as arbitrary U/mL for each analyte.

2.3.1. Bead Reagents

1. Flex Set beads: each capture bead species supplied as bead suspensions in buffered saline solution containing fetal bovine serum and 0.09% sodium azide.
2. Instrument setup beads: setup beads defining the four corners of the Flex Set bead clusters (A1, A8, F1, F9 positions) are supplied as single-bead suspensions and are used to adjust the optimal instrument setup.
3. PE instrument setup bead: suspension of F1 beads, used for generating a PE-positive bead population for instrument setup. The beads are covalently coupled with an anti-immunoglobulin antibody.

2.3.2. Antibody Reagents

1. Cell signaling Flex Set detectors: each analyte is defined by both the specific capture bead and the complementary PE-conjugated detector antibody reagent. The corresponding bead and detector specificities, together with lyophilized standards, are supplied for each analyte in a single package. The antibody conjugates are supplied in buffered saline solution containing BSA and 0.09% sodium azide.
2. PE-positive control detector: a single vial of PE-conjugated antibody that is formulated for use at 50 µL/test. This reagent is used with the PE instrument setup bead to set instrument compensation. Store at 4°C. Do not freeze.

2.3.3. Flex Set Standards

Each cell signaling flex set assay standard is formulated in aqueous solution containing 0.09% sodium azide. The standard is packaged together with the capture bead and the detector.

2.3.4. Buffers and Diluents

1. Wash buffer: 1X PBS solution containing protein and detergent used for wash steps and to resuspend beads for analysis. Store at 4°C.
2. Assay diluent: buffered solution used to dilute the cell signaling standards and to dilute test samples. Store at 4°C.
3. 5X Denaturation buffer: 5X sodium dodecyl sulfate solution used to denature test samples. Store at room temperature or 4°C.
4. Capture bead diluent: PBS solution containing protein. It is used to dilute capture beads prior to each experiment. Store at 4°C.
5. Detection reagent diluent: PBS solution containing protein. It is used to dilute the detection reagents prior to each experiment. Store at 4°C.

2.3.5. Instrumentation, Equipment, and Software

Same as described in **Subheading 2.2.5**.

3. Methods

3.1. Human Th1/Th2 Bead Assay

The human Th1/Th2 CBA Assay is used as a representative example of the ready-to-use CBA kits. **Table 1** summarizes the available kits and provides the list of analytes within each kit.

3.1.1. Preparation of Human Th1/Th2 Cytokine Standards

The human Th1/Th2 cytokine standards are lyophilized and should be reconstituted and serially diluted before mixing with the capture beads and the PE-detection reagent.

1. Reconstitute one vial of lyophilized human Th1/Th2 cytokine standards with 0.2 mL of assay diluent to prepare a 10X bulk standard. Allow the reconstituted standard to

equilibrate for at least 15 min before making dilutions. Agitate vial to mix thoroughly. Do not vortex.
2. Label 12 × 75-mm tubes (BD Falcon, cat. no. 352008) and arrange them in the following order: Top standard, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256.
3. Add 900 µL of assay diluent to the top standard tube.
4. Add 300 µL of assay diluent to each of the remaining tubes.
5. Transfer 100 µL of 10X bulk standard to the top standard tube and mix thoroughly.
6. Perform a serial dilution by transferring 300 µL from the top standard to the 1:2 dilution tube and mix thoroughly. Continue making serial dilutions by transferring 300 µL from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube and mix thoroughly. The assay diluent serves as the negative control.

3.1.2. Preparation of Mixed Human Th1/Th2 Cytokine Capture Beads

The capture beads are bottled individually, and it is necessary to pool the six individual bead reagents immediately before mixing them together with the PE-detection reagent, standards, and samples.

1. Determine the number of assay tubes (including standards and controls) that are required for the experiment (e.g., 8 unknowns, 9 cytokine standard dilutions, and 1 negative control = 18 assay tubes).
2. Vigorously vortex each capture bead suspension for a few seconds before mixing.
3. Add a 10-µL aliquot of each capture bead, for each assay tube to be analyzed, into a single tube labeled "mixed capture beads" (e.g., 10 µL of IL-2 capture beads × 18 assay tubes = 180 µL of IL-2 capture beads required).
4. Vortex the bead mixture thoroughly. The mixed capture beads are now ready to be transferred to the assay tubes.

3.1.3. Preparation of Test Samples

The standard curve for each cytokine covers a defined set of concentrations from 20 to 5000 pg/mL. It may be necessary to dilute test samples to ensure that their mean fluorescence values fall within the limits or range of the generated cytokine standard curve. For best results, samples that are known or assumed to contain high levels of a given cytokine should be diluted as described next.

1. Dilute test sample by the desired dilution factor (i.e., 1:2, 1:10, or 1:100) using the appropriate volume of assay diluent.
2. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing mixed capture beads and PE detection reagent.

3.1.4. Assay Procedure

Following the preparation and dilution of the standards and mixing of the capture beads, transfer these reagents and test samples to the appropriate assay tubes for incubation and analysis.

1. Add 50 µL of the mixed capture beads to the appropriate assay tubes. Vortex the mixed capture beads before adding to the assay tubes.

2. Add 50 µL of the human Th1/Th2 PE-detection reagent to the assay tubes.
3. Add 50 µL of the human Th1/Th2 cytokine standard dilutions to the control assay tubes.
4. Add 50 µL of each test sample to the test assay tubes.
5. Incubate the assay tubes for 3 h at room temperature and protect from direct exposure to light.
6. Add 1 mL of wash buffer to each assay tube and centrifuge at 200*g* for 5 min.
7. Carefully aspirate and discard the supernatant from each assay tube.
8. Add 300 µL of wash buffer to each assay tube to resuspend the bead pellet.
9. Begin analyzing samples on a flow cytometer. Vortex each sample for 3–5 s immediately before analyzing on the flow cytometer.

3.1.5. Data Acquisition and Data Analysis

Each assay tube is acquired on the flow cytometer using a CBA acquisition template. Acquisition template may be downloaded via the Internet from www.bdbiosciences.com/pharmingen/CBA//Dual-Laser.pdf. This template assures that each collected sample file contains approx 300 events for each capture bead species. To facilitate analysis of data files using the BD CBA software a numeric suffix is added to each file that corresponds to the assay tube number (i.e., tube no. 1 containing 0 pg/mL could be saved as RV032595.001). The acquired FACS files are saved and then analyzed using the BD CBA software. The outputs of the analysis are the calibration curves for the six analytes (**Fig. 2**) and tabulated concentrations of the six cytokines for each sample.

3.2. Soluble Human Protein Flex Set Bead Assay

3.2.1. Preparation of Human Soluble Protein Flex Set Standards

The two standards provided with each soluble protein Flex Set are provided as a 10X bulk recombinant protein (50,000 pg/mL) when reconstituted in 0.2 mL of assay diluent and should be serially diluted before mixing with the capture beads and the PE-detection reagent for a given assay. Each assay (single bead or multiplex) performed in a given experiment will need to have a standard curve prepared.

1. For multiplex experiments involving 10 or fewer soluble protein Flex Set assays, reconstitute each Flex Set standard vial with 0.2 mL of assay diluent to prepare a 10X bulk standard. Allow the reconstituted standard to equilibrate for at least 15 min before making dilutions. Mix reconstituted protein by pipet only. Do not vortex.
2. Label 12 × 75-mm tubes (BD Falcon, cat. no. 352008) and arrange them in the following order: top standard, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256.
3. Add 100 µL of each soluble protein standard to be run in the experiment to the top standard tube.

4. Add assay diluent to the top standard tube to bring the final volume to 1 mL.
   Example: if five soluble protein Flex Sets are being multiplexed for a given experiment, 100 µL of each soluble protein Flex Set standard needs to be added to the top standard tube (5 × 100 µL = 500 µL total volume) and then add 500 µL of assay diluent (1 mL assay diluent – 500 µL [volume of standards added] = 500 µL assay diluent). Adjust calculations accordingly for multiplexes of 10–20 soluble protein Flex Set assays.
5. Add 500 µL of assay diluent to each of the remaining tubes.
6. Perform a serial dilution by transferring 500 µL from the top standard to the 1:2 dilution tube and mix thoroughly. Continue making serial dilutions by transferring 500 µL from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube and mix thoroughly. Mix by pipet only, do not vortex. Prepare one tube containing assay diluent to serve as negative control.

3.2.2. Preparation of Test Samples

The standard curve for each soluble protein Flex Set covers a defined set of concentrations from 20 to 5000 pg/mL. It may be necessary to dilute test samples to ensure that their mean fluorescence values fall within the limits or range of the generated standard curve. For best results, samples that are known or assumed to contain high levels of a given protein should be diluted as described next.

1. Dilute test sample by the desired dilution factor (i.e., 1:10 or 1:100) using the appropriate volume of assay diluent. Serum or plasma samples must be diluted at least 1:4 before transferring the samples to the assay tubes or wells.
2. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing capture beads. Do not vortex. Mix by pipet only.

3.2.3. Preparation of Soluble Protein Flex Set Capture Beads

The capture beads provided with each soluble protein Flex Set are a 50X bulk (1 µL/test) and should be mixed with other soluble protein Flex Set capture beads and diluted to their optimal volume per test (50 µL/test) before adding the beads to a given tube or assay well.

1. Determine the number of soluble protein Flex Sets to be used in each tube or assay well in the experiment (size of the multiplex).
2. Determine the number of tubes or wells in the experiment.
3. Vortex each Soluble Protein Flex Set capture bead and then transfer 1 µL/test of each capture bead to a conical tube labeled "mixed capture beads."
   a. If testing cell culture supernatant samples, add capture bead diluent to the mixed capture beads tube to bring the final volume to 50 µL/test.
   b. If testing serum or plasma samples, add 0.5 mL of wash buffer to the mixed capture beads tube and centrifuge at 200*g* for 5 min to pellet the beads. Discard the supernatant by aspiration. Resuspend beads in capture bead diluent for serum/plasma to a final volume of 50 µL/test and incubate for 15 min at room temperature before proceeding to **step 5**.

Example: if five soluble protein Flex Sets are being multiplexed for a given 20 test experiment, you would add 1 µL/test of each capture bead to the mixed capture Bead tube (1 µL/test × 20 tests = 20 µL total volume of each soluble protein Flex Set capture bead) and then add capture bead diluent to bring the final volume to 50 µL/test by determining the remaining volume to add (the final volume of mixed capture beads is 20 tests × 50 µL/test = 1000 µL). A total of 100 µL of capture beads were added to the mixed capture beads tube previously listed when 20 µL total volume of each capture bead was added from the five soluble protein Flex Sets. The amount of capture bead diluent to add is 1000 µL total volume – 100 µL of capture beads = 900 µL).

5. Vortex the beads to mix thoroughly. Mixed capture beads are now ready to be used in the experiment.

3.2.4. Preparation of Flex Set PE-Detection Reagents

The PE-detection reagent provided with each soluble protein Flex Set is a 50X bulk (1 µL/test) and should be mixed with other soluble protein Flex Set PE-detection reagent and diluted to their optimal volume per test (50 µL/test) before adding the PE-detection reagents to a given tube or assay well.

1. Determine the number of soluble protein Flex Sets to be used in each tube or assay well in the experiment (size of the multiplex).
2. Determine the number of assay tubes or wells to be run in the experiment.
3. Transfer 1 µL/test of each soluble protein Flex Set PE-detection reagent to a conical tube labeled "mixed PE-detection reagent."
4. Add detection reagent diluent to the mixed PE-detection reagent tube to bring the final volume to 50 µL/test.
   Example: if five soluble protein Flex Sets are being multiplexed for a given 20 test experiment, you would add 1 µL/test of each soluble protein Flex Set PE-detection reagent to the mixed PE-detection reagent tube (1 µL/test × 20 tests = 20 µL total volume of each PE-detection reagent) and then add detection reagent diluent to bring the final volume to 50 µL/test by determining the remaining volume to add (the final volume of mixed PE-detection reagent is 20 tests × 50 µL/test = 1000 µL). A total of 100 µL of PE-detection reagent was added to the mixed PE-detection reagent tube previously listed when 20 µL total volume of each PE-detection reagent was added from the five soluble protein Flex Sets. The amount of detection reagent diluent to add is 1000 µL total volume – 100 µL of PE-detection reagents = 900 µL).
5. Vortex mixed PE-detection reagent briefly. Mixed PE-detection reagent is now ready to be used in the experiment.

3.2.5. Soluble Protein Flex Set Assay Procedure

Transfer the standards, capture beads, test samples, and PE-detection reagent to the appropriate assay tubes or wells for incubation and analysis.

1. For assays performed in filter plates, prewet the plate by adding 100 µL of wash buffer to each well. To remove excess volume, apply plate to vacuum manifold.

Do not exceed 10 inches of Mercury vacuum pressure 500*g*. Do not aspirate until wells are dry, leave a small amount of wash buffer in the wells.
2. Add 50 µL of the mixed capture beads to the appropriate assay tubes or wells. Vortex the mixed capture beads before adding them to the assay tubes or wells.
3. Add 50 µL of the soluble protein Flex Set standard dilutions to the control assay tubes or wells.
4. Add 50 µL of each test sample to the test assay tubes or wells.
5. For assays performed in tubes, mix assay tubes gently and incubate for 1 h at room temperature and protect from direct exposure to light. For assays performed in filter plate wells, mix the microwell plate for 5 min using a digital shaker at 500 and incubate plate for 1 h at room temperature, protecting from direct exposure to light.
6. Add 50 µL of the mixed PE-detection reagent to the assay tubes or wells.
7. For assay performed in tubes, mix assay tubes gently and incubate for 2 h at room temperature and protect from direct exposure to light. For assays performed in filter plate wells, mix the microwell plate for 5 min using a digital shaker at 50*g* and incubate plate for 2 h at room temperature, protecting from direct exposure to light.
8. For assays run in tubes, add 1.0 mL of wash buffer to each assay tube and centrifuge at 200*g* for 5 min. For assays run in filter plate wells, apply the plate to the vacuum manifold and vacuum aspirate (do not exceed 10" Hg of vacuum pressure) until wells are drained (2–10 s).
9. For assays run in tubes, carefully aspirate and discard the supernatant from each assay tube. For assays run in filter plate wells, proceed to **step 10**.
10. Add 300 µL of wash buffer to each assay tube or 150 µL of wash buffer to each assay well. Vortex assay tubes briefly or shake microwell plate on a digital shaker at 500*g* for 5 min to resuspend beads.
11. Begin analyzing samples on a flow cytometer. For assays run in tubes, it is recommended that each tube be vortexed briefly before analyzing on the flow cytometer.

3.2.6. Instrument Setup, Data Acquisition, and Analysis

FACSComp software is used for the daily setup the FACSCalibur flow cytometer. CellQuest software is required for analyzing samples and formatting data for subsequent analysis using the FCAP Array software. Setup for the FACSArray bioanalyzer is required only once a month. Sample acquisition is automated on the FACSCalibur, using the carousel loader, whereas the FACSArray instrument acquires the samples directly from the microplate wells. The data are analyzed using the FCAP Array software. The outputs of the Flex Set assays are the standard curves for each assay (**Fig. 5**) and the tabulated results for each analytes.

3.3. Cell Signaling Bead Assay

3.3.1. Preparation of Cell Signaling Flex Set Standards

The standard provided with each Cell Signaling Flex Set is provided as a 50X bulk recombinant protein (50,000 U/mL) and should be serially diluted

before mixing with the capture beads and the PE-detection reagent for a given assay. The protocol listed next indicates how standards should be mixed and diluted for use in a cell signaling Flex Set assay. Each assay (single bead or multiplex) performed in a given experiment will need to have a standard curve prepared. Each cell signaling Flex Set standard was assigned an arbitrary unit value. In each case, the unit potency of the Flex Set standard will be kept consistent from lot to lot.

1. Warm standard vial to 37°C and vortex to mix thoroughly.
2. Label 12 × 75-mm tubes (BD Falcon, cat. no. 352008) and arrange them in the following order: top standard, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256.
3. Add 20 µL of each cell signaling Flex Set standard to be run in the experiment to the top standard tube.
4. Add assay diluent to the top standard tube to bring the final volume to 1 mL.
   Example: if five cell signaling Flex Sets are being multiplexed for a given experiment, 20 µL of each BD CBA cell signaling flex.
   Set standard needs to be added to the top standard tube (5 × 20 µL = 100 µL total volume) and will then add 900 µL of assay diluent (1 mL assay diluent – 100 µL [volume of standards added] = 900 µL assay diluent).
5. Add 500 µL of assay diluent to each of the remaining tubes.
6. Perform a serial dilution by transferring 500 µL from the top standard to the 1:2 dilution tube and mix thoroughly. Continue making serial dilutions by transferring 500 µL from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube and mix thoroughly. The assay diluent serves as the negative control.

3.3.2. Preparation of Test Samples

The cell signaling Flex Sets are designed to measure total or phosphorylated proteins from denatured cell lysate samples. The tested cells need to be lysed and denatured using the 5X denaturation buffer (provided in the kit) before used in a Flex Set assay. The standard curve for each Flex Set covers a defined set of concentrations between 3.9 and 1000 U/mL. It may be necessary to dilute test samples to ensure that their mean fluorescence values fall within the limits or range of the generated standard curve. For best results, samples that are known or assumed to contain high levels of a given protein should be diluted. In cases where the samples are known or assumed to contain low levels of a given protein, the sample should be lysed in a lower volume of lysis buffer thereby concentrating the protein in the sample. It is important that the cell number or the total protein concentration of the cell lysate sample is known so that results determined using the Flex Sets can be normalized (e.g., U/mL/10^6 cells or U/mL/µg of cell lysate). It is necessary to heat the 5X denaturation buffer to 37°C before use (shake or vortex until all precipitates have gone back into solution). The final concentration of the denaturation buffer should reach 1X once mixed with cells to achieve denaturation of the cell lysate.

3.3.2.1. Cells in Suspension

1. Count cells in sample. This is to give an approximate idea of protein concentration, which should be greater than 1 mg/mL (protein concentration is dependent on cell type, e.g., Jurkat = 100 – 200 μg/10^6 cells whereas peripheral blood lymphocytes = 25 – 50 μg/10^6 cells).
2. Treat cells to induce or inhibit protein phosphorylation as required for the experiment.
3. Add appropriate amount of 5X denaturation buffer so that the final concentration is 1X. Alternatively, ice-cold PBS can be added to the tube and the cells pelleted. Add an appropriate amount of 1X denaturation buffer (prepared by diluting the 5X denaturation buffer with water) to resuspend the cell pellet.
4. Immediately place in a boiling water bath for 5 min.
5. Determine protein concentration.
6. Dilute cell lysate sample by the desired dilution factor (i.e., 1:2, 1:10, or 1:20) using the appropriate volume of assay diluent. Sample must be diluted at least 1:2 to reduce the percentage of sodium dodecyl sulfate.
7. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing capture beads.

3.3.2.2. Adherent Cells

1. Count cells before plating. This is to give an approximate idea of protein concentration, which should be greater than 1 mg/mL.
2. Treat cells to induce or inhibit protein phosphorylation as required for the experiment.
3. Add the appropriate amount of 5X denaturation buffer so that the final concentration is 1X. Alternatively, aspirate off all liquid and add denaturation buffer diluted to 1X with water. Scrape or agitate cells to dislodge from plate.
4. Immediately place in a boiling water bath for 5 min.
5. Determine protein concentration.
6. Dilute cell lysate sample by the desired dilution factor (i.e., 1:2, 1:10, or 1:20) using the appropriate volume of assay diluent. Sample must be diluted at least 1:2.
7. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing capture beads.

3.3.3. Preparation of Capture Beads

The capture beads provided with each cell signaling Flex Set are a 50X bulk (1 μL/test) and should be mixed with other cell signaling Flex Set capture beads and diluted to their optimal volume per test (50 μL/test) before adding the beads to a given tube or assay well.

1. Determine the number of BD CBA cell signaling Flex Sets to be used in each tube or assay well in the experiment (size of the multiplex).
2. Determine the number of assay of tubes or wells to be run in the experiment.

3. Vortex each cell signaling Flex Set capture bead and then transfer 1 µL/test of each cell signaling Flex Set capture bead to a conical tube labeled "mixed capture beads."
4. Add capture bead diluent to the mixed capture beads tube to bring the final volume to 50 µL/test.
   Example: if five cell signaling Flex Sets are being multiplexed for a given 20 test experiment, 1 µL/test of each cell signaling Flex Set capture bead needs to be added to the mixed capture bead tube (1 µL/test × 20 tests = 20 µL total volume of each cell signaling Flex Set capture bead) and then capture bead diluent is added to bring the final volume to 50 µL/test by determining the remaining volume to add (the final volume of mixed capture beads is 20 tests × 50 µL/test = 1000 µL). A total of 100 µL of capture beads were added to the mixed capture beads tube previously listed when 20 µL total volume of each cell signaling Flex Set capture bead was added from the five cell signaling Flex Sets. The amount of capture bead diluent to add is 1000 µL total volume – 100 µL of capture beads = 900 µL).
5. Vortex the beads to mix thoroughly. Mixed capture beads are now ready to be used in the experiment

3.3.4. Preparation of PE-Detection Reagents

The PE-detection reagent provided with each cell signaling Flex Set is a 50X bulk (1 µL/test) and should be mixed with other cell signaling Flex Set PE-detection reagent and diluted to their optimal volume per test (50 µL/test) before adding the PE-detection reagents to a given tube or assay well.

1. Determine the number of cell signaling Flex Sets to be used in each tube or assay well in the experiment (size of the multiplex).
2. Determine the number of assay tubes or wells to be run in the experiment.
3. Transfer 1 µL/test of each cell signaling Flex Set PE-detection reagent to a conical tube labeled "mixed PE-detection reagent."
4. Add detection reagent diluent to the mixed PE-detection reagent tube to bring the final volume to 50 µL/test.
   Example: if five cell signaling Flex sets are being multiplexed for a given 20 test experiment, 1 µL/test of each cell signaling Flex Set PE-detection reagent needs to be added to the mixed PE-detection reagent tube (1 µL/test × 20 tests = 20 µL total volume of each BD CBA cell signaling Flex Set PE-detection reagent) and then add detection reagent diluent to bring the final volume to 50 µL/test by determining the remaining volume to add (the final volume of mixed PE-detection reagent is 20 tests × 50 µL/test = 1000 µL). A total of 100 µL of PE-detection reagent was added to the mixed PE-detection reagent tube previously listed when 20 µL total volume of each cell signaling Flex Set PE-detection reagent was added from the five cell signaling Flex Sets. The amount of detection reagent diluent to add is 1000 µL total volume – 100 µL of PE-detection reagents = 900 µL.
5. Vortex mixed PE-detection reagent briefly. Mixed PE-detection reagent is now ready to be used in the experiment.

3.3.5. Assay Procedure

Transfer the standards, capture beads, test samples, and PE-detection reagent to the appropriate assay tubes or wells for incubation and analysis. Flex Set standards are run in each experiment to allow quantitation of test samples.

1. Add 50 µL of the mixed capture beads to the appropriate assay tubes or wells. Vortex the mixed capture beads before adding them to the assay tubes or wells.
2. Add 50 µL of the mixed PE-detection reagent to the assay tubes or wells.
3. Add 50 µL of the cell signaling Flex Set standard dilutions to the control assay tubes or wells.
4. Add 50 µL of each denatured cell lysate test sample to the test assay tubes or wells.
5. For assays performed in tubes, mix assay tubes gently and incubate for 4 h at room temperature and protect from direct exposure to light. For assays performed in filter plate wells, mix the microwell plate for 15 min using a digital shaker at 500*g* and incubate plate for 4 h at room temperature and protect from direct exposure to light.
6. For assays run in tubes, add 1.0 mL of wash buffer to each assay tube and centrifuge at 200*g* for 5 min. For assays run in filter plate wells, apply the plate to the vacuum manifold and vacuum aspirate (do not exceed 10" Hg of vacuum) until wells are drained (2–10 s).
7. For assays run in tubes, carefully aspirate and discard the supernatant from each assay tube. For assays run in filter plate wells, proceed to **step 8**.
8. Add 300 µL of wash buffer to each assay tube or 150 µL of wash buffer to each assay well. Vortex assay tubes briefly or shake microwell plate on a digital shaker at 50*g* for 5 min to resuspend beads.
9. Begin analyzing samples on a flow cytometer. For assays run in tubes, it is recommended that each tube be vortexed briefly before analyzing on the flow cytometer.

3.3.6. Instrument Setup, Data Acquisition, and Analysis

FACSComp software is used for setting up the FACSCalibur flow cytometer daily. BD CellQuest software is required for analyzing samples and formatting data for subsequent analysis using the FCAP Array software. Setup for the FACSArray Bioanalyzer required only once a month. Sample acquisition is automated on the FACSCalibur, using the carousel loader, whereas the FACSArray instrument acquires the samples directly from the microplate wells. The data are analyzed using the FCAP Array Software. The outputs of the Flex Set assays are the standard curves for each assay and the tabulated results for each analytes. **Fig. 6** shows the bead positions of a 9-plex cell signaling Flex Set combination. **Fig. 7** demonstrates the kinetics of T cell activation with CD3/CD28 treatment using Jurkat cells.

4. Notes

1. The BD CBA is not recommended for use on stream-in-air instruments where signal intensities may be reduced, adversely affecting assay sensitivity. Stream-in-air instruments include the FACStar Plus and FACSVantage flow cytometers.
2. The antibody-conjugated beads will settle out of suspension over time. It is necessary to vortex the vial vigorously for 3–5 s before taking a bead suspension aliquot.
3. The human Th1/Th2 cytokine standards vials are stable until the kit expiration date. Following reconstitution, store the freshly reconstituted 10X bulk standard at 2–8°C and use within 12 h.
4. When running experiments with higher order multiplexes use the following instructions for reconstituting the soluble protein Flex Set standards. For multiplex experiments involving 10–20 soluble protein Flex Set assays, reconstitute each standard vial with 0.1 mL of assay diluent to prepare a 20X bulk standard. For multiplex experiments involving more than 20 soluble protein Flex Set assays, pour each standard protein sphere into a 15-mL conical tube and reconstitute all spheres together in 2 mL of assay diluent to prepare a top standard mixture.
5. To calibrate the flow cytometer and quantitate test samples, it is necessary to run the cytokine standards and the cytometer setup controls in each experiment.
6. For Flex Set assays that will be acquired on a FACSCalibur flow cytometer, it is recommended that additional dilutions of the standard be prepared (i.e., 1:512 and 1:1024) as it is possible that in multiplex experiments containing a large number of assays, the top standard and 1:2 standard dilution will not be analyzable by the FCAP Array software. In those cases, the top standard and 1:2 standard dilutions can be run on the experiment but will be excluded from the final analysis in the FCAP Array software.
7. Cell lysates may be stored at –70°C for up to 6 mo. Multiple freeze/thaw treatments of sample should be avoided.
8. It is necessary to analyze CBA samples on the day of the experiment. Prolonged storage of samples, once the assay is complete, can lead to increased background and reduced sensitivity.
9. The phospho-specific cell signaling Flex Set assays cannot be used in the same assay well with the total protein cell signaling Flex Set assays. An updated assay compatibility chart for the cell signaling Flex Sets is available at www.bdbiosciences.com/flexset.

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