

BD™ Cytometric Bead Array (CBA)

# Human Inflammation Kit

## Instruction Manual

Cat. No. 551811



BD Biosciences

*For Research Use Only. Not for use in diagnostic or therapeutic procedures. Purchase does not include or carry any right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of Becton Dickinson and Company is strictly prohibited.*

BD, BD Logo and all other trademarks are the property of Becton, Dickinson and Company. ©2003 BD

# Kit Contents

(Store the following items at 4°C)

- A1 Human IL-8 Capture Beads: 1 vial, 0.8 ml
- A2 Human IL-1 $\beta$  Capture Beads: 1 vial, 0.8 ml
- A3 Human IL-6 Capture Beads: 1 vial, 0.8 ml
- A4 Human IL-10 Capture Beads: 1 vial, 0.8 ml
- A5 Human TNF Capture Beads: 1 vial, 0.8 ml
- A6 Human IL-12p70 Capture Beads: 1 vial, 0.8 ml
- B Human Inflammation PE\* Detection Reagent: 1 vial, 4 ml
- C Human Inflammation Standards: 2 vials, 0.2 ml lyophilized
- D Cytometer Setup Beads: 1 vial, 1.5 ml
- E1 PE Positive Control Detector: 1 vial, 0.5 ml
- E2 FITC Positive Control Detector: 1 vial, 0.5 ml
- F Wash Buffer: 2 bottles, 130 ml
- G Assay Diluent: 1 bottle, 30 ml

Patents: \*US 4,520,110, Europe 76,695, Canada, 1,179,942

*Unless otherwise specified, all products are for Research Use Only.  
Not for use in diagnostic or therapeutic procedures. Not for resale.*

# Table of Contents

Introduction . . . . .	6
Principle of the Test . . . . .	7
Advantages . . . . .	7
Limitations . . . . .	8
Reagents Provided . . . . .	8
Bead Reagents . . . . .	8
Antibody and Standard Reagents . . . . .	9
Buffer Reagents . . . . .	9
Materials Required but not Provided . . . . .	10
Overview: Human Inflammation CBA Assay Procedures . . . . .	11
Culture Supernatant Assay Procedure . . . . .	11
Serum/Plasma Assay Procedure . . . . .	12
Preparation of Human Inflammation Standards . . . . .	12
Preparation of Mixed Human Inflammation Capture Beads . . . . .	14
Preparation of Test Samples . . . . .	14
Human Inflammation CBA Assay Procedures . . . . .	14
Culture Supernatant Assay Procedure . . . . .	15
Serum/Plasma Assay Procedure . . . . .	15
Cytometer Setup, Data Acquisition, and Analysis. . . . .	16
Preparation of Cytometer Setup Beads . . . . .	16
Instrument Setup with BD FACSCComp™ Software and BD CaliBRITE™ Beads . . . . .	17
Instrument Setup with the Cytometer Setup Beads . . . . .	17
Data Acquisition . . . . .	20
Analysis of Sample Data . . . . .	22
Typical Data . . . . .	22

## Table of Contents *(continued)*

Performance . . . . .	24
Sensitivity . . . . .	24
Recovery . . . . .	25
Linearity . . . . .	26
Specificity . . . . .	27
Precision . . . . .	28
Troubleshooting Tips . . . . .	29
References. . . . .	30

## Introduction

Flow cytometry is an analysis tool that allows for the discrimination of different particles on the basis of size and color. Multiplexing is the simultaneous assay of many analytes in a single sample. The BD™ Cytometric Bead Array (CBA) employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes. The BD CBA is combined with flow cytometry to create a powerful multiplexed assay.

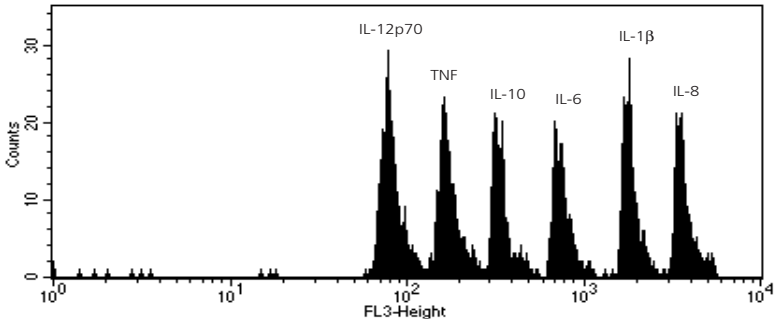
The BD CBA system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. Each bead in a CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate. The BD CBA capture bead mixture is in suspension to allow for the detection of multiple analytes in a small volume sample. The combined advantages of the broad dynamic range of fluorescent detection via flow cytometry and the efficient capturing of analytes via suspended particles enable CBA to use fewer sample dilutions and to obtain the value of an unknown in substantially less time (compared to conventional ELISA).

The BD Human Inflammation CBA Kit can be used to quantitatively measure Interleukin-8 (IL-8), Interleukin-1 $\beta$  (IL-1 $\beta$ ), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor (TNF), and Interleukin-12p70 (IL-12p70) protein levels in a single sample. The kit performance has been optimized for analysis of specific proteins in tissue culture supernatants, EDTA plasma, and serum samples using one of two protocols depending on sample source.

The BD CBA System, a product of BD Biosciences, was developed jointly by BD Biosciences Immunocytometry Systems and BD Biosciences Pharmingen. This kit incorporates the quality, reliability, and service that you have learned to expect from BD Biosciences.

# Principle of the Test

Six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-8, IL-1 $\beta$ , IL-6, IL-10, TNF, and IL-12p70 proteins. The six bead populations are mixed together to form the CBA which is resolved in the FL3 channel of a flow cytometer such as the BD FACScan™ or BD FACSCalibur™ flow cytometer.



**Figure 1**

The capture beads, PE-conjugated detection antibodies, and recombinant standards 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 graphical and tabular format using the BD CBA Analysis Software. The kit provides sufficient reagents for the quantitative analysis of 50 test samples and the generation of two standard curve sets.

## Advantages

The BD CBA provides several advantages when compared with conventional ELISA methodology:

- The required sample volume is approximately one-sixth the quantity necessary for conventional ELISA assays due to the detection of six analytes in a single sample.
- A single set of diluted standards is used to generate a standard curve for each analyte.
- A CBA experiment takes less time than a single ELISA and provides results that would normally require six conventional ELISAs.

## Limitations

The sensitivity of the Human Inflammation CBA is comparable to conventional ELISA, but due to the complexity and kinetics of this multi-analyte assay, actual sensitivity in a given experiment may vary slightly (see *Sensitivity* and *Precision* information on page 24 and 28).

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

Serum spike recoveries for IL-1 $\beta$ , TNF, and IL-12p70 are lower than for the other proteins in this assay. This variation is due to assay conditions and serum proteins and may affect quantitation of these proteins in serum samples.

The sensitivity for the detection of IL-1 $\beta$  in this assay is less than for the other proteins measured. It is possible that, due to operator variation, instrument settings, and instrument performance, the 20 pg/ml standard curve point for IL-1 $\beta$  may not have signal intensity above the 0 pg/ml background level.

## Reagents Provided

### Bead Reagents

**Human Inflammation Capture Beads (A1 – A6):** The specific capture beads, having discrete fluorescence intensity characteristics, are distributed from brightest to dimmest as follows:

Bead	Specificity
<b>(Brightest) A1</b>	<b>IL-8</b>
<b>A2</b>	<b>IL-1<math>\beta</math></b>
<b>A3</b>	<b>IL-6</b>
<b>A4</b>	<b>IL-10</b>
<b>A5</b>	<b>TNF</b>
<b>(Dimmest) A6</b>	<b>IL-12p70</b>

A single 75-test vial of each specific capture bead (A1 – A6) is included in this kit. Store at 4°C. Do not freeze.

*Note:* The antibody-conjugated beads will settle out of suspension over time. It is necessary to vortex the vial vigorously for 3 – 5 seconds before taking a bead suspension aliquot.

**Cytometer Setup Beads (D):** A single 30-test vial of setup beads for setting the initial instrument PMT voltages and compensation settings is sufficient for 10 instrument setup procedures. The Cytometer Setup Beads are formulated for use at 50  $\mu$ l/test.



## Antibody and Standard Reagents

**Human Inflammation PE Detection Reagent (B):** A 75-test vial of PE-conjugated anti-human IL-8, IL-1 $\beta$ , IL-6, IL-10, TNF, and IL-12p70 antibodies that is formulated for use at 50  $\mu$ l/test. Store at 4°C. Do not freeze.

**PE Positive Control Detector (E1):** A 10-test vial of PE-conjugated antibody control that is formulated for use at 50  $\mu$ l/test. This reagent is used with the Cytometer Setup Beads to set the initial instrument compensation settings. Store at 4°C. Do not freeze.

**FITC Positive Control Detector (E2):** A 10-test vial of FITC-conjugated antibody control that is formulated for use at 50  $\mu$ l/test. This reagent is used with the Cytometer Setup Beads to set the initial instrument compensation settings. Store at 4°C. Do not freeze.

**Human Inflammation Standards (C):** Two vials containing lyophilized recombinant human proteins. Each vial should be reconstituted in 0.2 ml of Assay Diluent to prepare a 10 $\times$  bulk standard. The reconstituted 10 $\times$  bulk standard contains 50 ng/ml of each recombinant human IL-8, IL-1 $\beta$ , IL-6, IL-10, TNF, and IL-12p70 protein. Store at 4°C.

*Note:* The Human Inflammation Standards vials are stable until the kit expiration date. Following reconstitution, store the freshly reconstituted 10 $\times$  bulk standard at 2 – 8°C and use within 12 hours.

## Buffer Reagents

**Assay Diluent (G):** A single 30 ml bottle of a buffered protein\* solution (1 $\times$ ) used to reconstitute and dilute the Human Inflammation Standards and to dilute test samples. Store at 4°C.

**Wash Buffer (F):** Two 130 ml bottles of phosphate buffered saline (PBS) solution (1 $\times$ ), containing protein\* and detergent, used for wash steps and to resuspend the washed beads for analysis. Store at 4°C.

### *Hazardous Ingredients:*

#### Sodium Azide:

Component D contains 0.1% sodium azide. Components A1 - A6, B, E1 - E2, F, and G contain 0.09% sodium azide. Sodium azide yields a highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discharging to avoid accumulation of potentially explosive deposits in plumbing.

\*Source of all serum proteins is from the United States

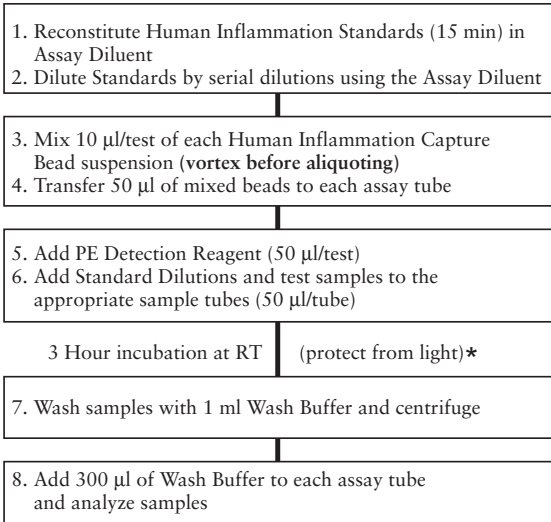
## Materials Required but not Provided

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

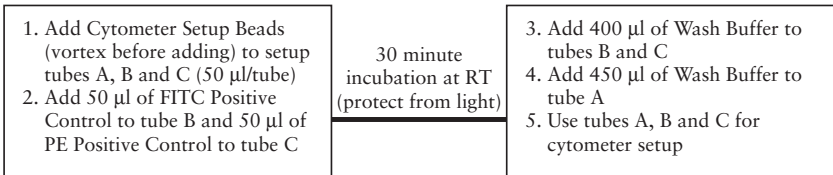
- A flow cytometer equipped with a 488 nm laser capable of detecting and distinguishing fluorescence emissions at 576 and 670 nm (eg, BD FACScan™ or BD FACSCalibur™ instruments) and BD CellQuest™ Software.
- 12 × 75 mm sample acquisition tubes for a flow cytometer (eg, BD Falcon™ Cat. No. 352008).
- 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.
- BD CaliBRITE™ 3 Beads, (Cat. No. 340486).

# Overview: Human Inflammation CBA Assay Procedures

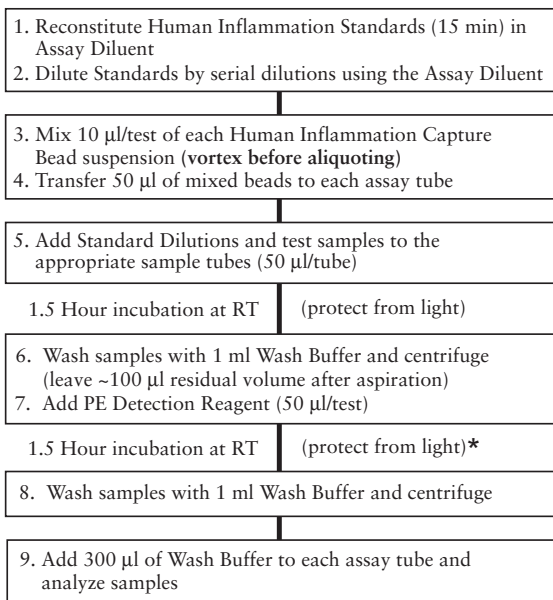
## Culture Supernatant Assay Procedure



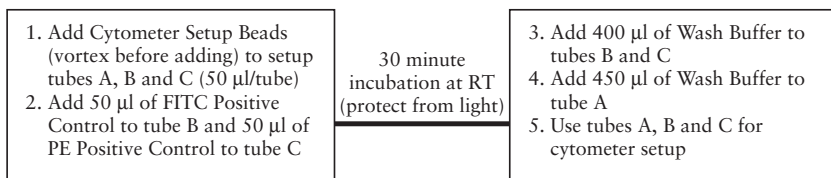
### \*Cytometer Setup Bead Procedure



## Serum/Plasma Assay Procedure



### \*Cytometer Setup Bead Procedure

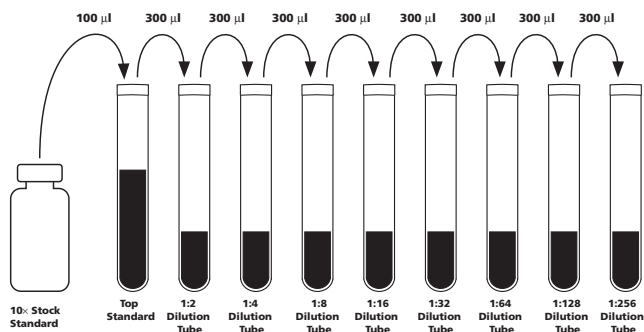


## Preparation of Human Inflammation Standards

The Human Inflammation Standards are lyophilized and should be reconstituted and serially diluted before mixing with the Capture Beads and the PE Detection Reagent.

1. Reconstitute 1 vial of lyophilized Human Inflammation Standards with 0.2 ml of Assay Diluent to prepare a 10× bulk standard. Allow the reconstituted standard to equilibrate for at least 15 minutes before making dilutions. Agitate vial 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 900 µl of Assay Diluent to the Top Standard tube.

4. Add 300  $\mu\text{l}$  of Assay Diluent to each of the remaining tubes.
5. Transfer 100  $\mu\text{l}$  of 10 $\times$  bulk standard to the Top Standard tube and mix thoroughly.
6. Perform a serial dilution by transferring 300  $\mu\text{l}$  from the Top Standard to the 1:2 dilution tube and mix thoroughly. Continue making serial dilutions by transferring 300  $\mu\text{l}$  from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube and mix thoroughly (see *Figure 2*.) The Assay Diluent serves as the negative control.



**Figure 2.** Preparation of Human Inflammation Standard Dilutions

The approximate concentration (pg/ml) of recombinant protein in each dilution tube is shown in Table 1.

**Table 1.** Human Inflammation Standard concentrations after dilution

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-8	5000	2500	1250	625	312.5	156	80	40	20
Human IL-1 $\beta$	5000	2500	1250	625	312.5	156	80	40	20
Human IL-6	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	5000	2500	1250	625	312.5	156	80	40	20
Human IL-12p70	5000	2500	1250	625	312.5	156	80	40	20

## Preparation of Mixed Human Inflammation Capture Beads

The Capture Beads are bottled individually, and it is necessary to pool the bead reagents (A1 – A6) immediately before mixing them together with the PE Detection Reagent, standards, or samples.

1. Determine the number of assay tubes (including standards and controls) that are required for the experiment (eg, 8 unknowns, 9 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  $\mu$ l aliquot of each Capture Bead, for each assay tube to be analyzed, into a single tube labeled “mixed Capture beads” (eg, 10  $\mu$ l of IL-8 Capture Beads  $\times$  18 assay tubes = 180  $\mu$ l of IL-8 Capture Beads required).
4. Vortex the Bead mixture thoroughly.

The mixed Capture beads are now ready to be transferred to the assay tubes (50  $\mu$ l of mixed Capture beads/tube) as described in *Human Inflammation CBA Assay Procedures*, page 14.

*Note:* Discard excess mixed Capture Beads. Do not store after mixing.

## Preparation of Test Samples

The standard curve for each protein covers a defined set of concentrations from 20 – 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 below.

1. Dilute test sample by the desired dilution factor (ie, 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.

## Human Inflammation CBA Assay Procedures

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. The serum/plasma assay procedure (page 15) should be used for any experiment testing serum or plasma samples. The serum/plasma assay procedure also works for culture supernatant. **In order to calibrate the flow cytometer and quantitate test samples, it is necessary to run the Inflammation Standards and the Cytometer Setup controls in each experiment.** See *Table 2* for a detailed description of the reagents added to the Inflammation Standard control assay tubes. The Cytometer Setup procedure is described on page 16.

## Culture Supernatant Assay Procedure

1. Add 50  $\mu$ l of the mixed Capture beads to the appropriate assay tubes. Vortex the mixed Capture beads before adding to the assay tubes.
2. Add 50  $\mu$ l of the Human Inflammation PE Detection Reagent to the assay tubes.
3. Add 50  $\mu$ l of the Human Inflammation Standard dilutions to the control assay tubes.
4. Add 50  $\mu$ l of each test sample to the test assay tubes.
5. Incubate the assay tubes for 3 hours at RT and protect from direct exposure to light. During this incubation, perform the Cytometer Setup procedure described on pages 16 and 17.
6. Add 1 ml of Wash Buffer to each assay tube and centrifuge at  $200 \times g$  for 5 minutes.
7. Carefully aspirate and discard the supernatant from each assay tube.
8. Add 300  $\mu$ l 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 seconds immediately before analyzing on the flow cytometer.**

## Serum/Plasma Assay Procedure

1. Add 50  $\mu$ l of the mixed Capture beads to the appropriate assay tubes. Vortex the mixed Capture beads before adding to the assay tubes.
2. Add 50  $\mu$ l of the Human Inflammation Standard dilutions to the control assay tubes.
3. Add 50  $\mu$ l of each test sample to the test assay tubes.
4. Incubate the assay tubes for 1.5 hours at RT and protect from direct exposure to light.
5. Add 1 ml of Wash Buffer to each assay tube and centrifuge at  $200 \times g$  for 5 minutes.
6. Carefully aspirate and discard the supernatant, leaving approximately 100  $\mu$ l of liquid in each assay tube. Aspiration should be done as consistently as possible.
7. Add 50  $\mu$ l of the Human Inflammation PE Detection Reagent to the assay tubes. Gently agitate assay tubes to resuspend bead pellet.
8. Incubate the assay tubes for 1.5 hours at RT and protect from direct exposure to light. During this incubation, perform the Cytometer Setup procedure described on pages 16 and 17.
9. Add 1 ml of Wash Buffer to each assay tube and centrifuge at  $200 \times g$  for 5 minutes.

10. Carefully aspirate and discard the supernatant from each assay tube.
11. Add 300  $\mu$ l of Wash Buffer to each assay tube to resuspend the bead pellet.
12. Begin analyzing samples on a flow cytometer. **Vortex each sample for 3 – 5 seconds immediately before analyzing on the flow cytometer.**

*Note:* 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.

**Table 2. Essential control assay tubes**

<b>Tube No.</b>	<b>Reagents (All reagents volumes are 50 <math>\mu</math>l)</b>
1 (Negative Control 0 pg/ml Standards)	mixed Capture beads, PE Detection Reagent, Assay Diluent
2 (20 pg/ml Standards)	mixed Capture beads, PE Detection Reagent, Inflammation Standards 1:256 Dilution
3 (40 pg/ml Standards)	mixed Capture beads, PE Detection Reagent, Inflammation Standards 1:128 Dilution
4 (80 pg/ml Standards)	mixed Capture beads, PE Detection Reagent, Inflammation Standards 1:64 Dilution
5 (156 pg/ml Standards)	mixed Capture beads, PE Detection Reagent, Inflammation Standards 1:32 Dilution
6 (312 pg/ml Standards)	mixed Capture beads, PE Detection Reagent, Inflammation Standards 1:16 Dilution
7 (625 pg/ml Standards)	mixed Capture beads, PE Detection Reagent, Inflammation Standards 1:8 Dilution
8 (1250 pg/ml Standards)	mixed Capture beads, PE Detection Reagent, Inflammation Standards 1:4 Dilution
9 (2500 pg/ml Standards)	mixed Capture beads, PE Detection Reagent, Inflammation Standards 1:2 Dilution
10 (5000 pg/ml Standards)	mixed Capture beads, PE Detection Reagent, Inflammation Standards "Top Standard"

## Cytometer Setup, Data Acquisition, and Analysis

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.

### Preparation of Cytometer Setup Beads

1. Add 50  $\mu$ l of Cytometer Setup Beads to three cytometer setup tubes labeled A, B, and C.
2. Add 50  $\mu$ l of FITC Positive Control Detector to tube B.
3. Add 50  $\mu$ l 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  $\mu$ l of Wash Buffer to tube A and 400  $\mu$ l of Wash Buffer to tubes B and C.
6. Proceed to next section.



## 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 CBA Instrument Setup template.

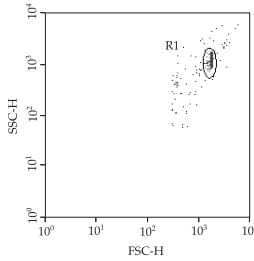
**Note:** The BD CBA Instrument Setup template can be found on the BD CBA software or BD FACStation™ CD for Macintosh computers in the BD CBA folder. Following installation on Macintosh computers using BD CBA Software Version 1.0, the template can be found in the BD Applications/BD CBA folder/Sample 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 may also be downloaded via the internet from: [www.bdbiosciences.com/pharmingen/CBA/downloads.shtml](http://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 FACSComp set.
5. Set the Threshold to FSC at 650.
6. In setup mode, run Cytometer Setup Beads tube A. Follow the setup instructions on pages 18 – 19.

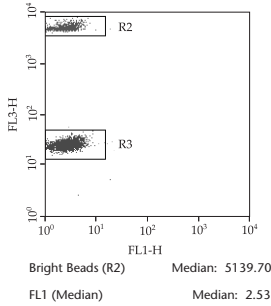
**Note:** Pause and restart acquisition frequently during the instrument setup procedure in order to reset detected values after settings adjustments.

Adjust gate R1 so that the singlet bead population is located in gate R1 (*Figure 3a*).



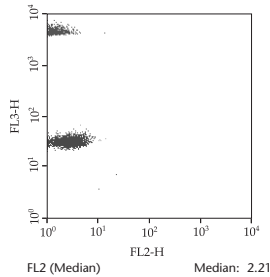
**Figure 3a**

Adjust the FL3 PMT so that the median of the top FL3 bead population's intensity is around 5000 (*Figure 3b*). Adjust gate R3 as necessary so that the dim FL3 bead population is located in gate R3 (*Figure 3b*). Do not adjust the R2 gate.



**Figure 3b**

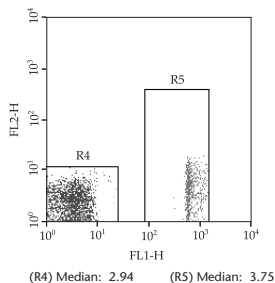
Adjust the FL1 PMT so that the median of FL1 is approximately 2.0 – 2.5 (*Figure 3b*). Adjust the FL2 PMT value so that the median of FL2 is approximately 2.0 – 2.5 (*Figure 3c*).



**Figure 3c**

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

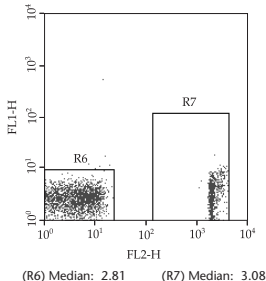
Adjust gate R5 as necessary so that the FL1 bright bead population is located in gate R5 (*Figure 3d*). Using the FL2 – %FL1 control, adjust the median of R5 to equal the median of R4 (*Figure 3d*).



**Figure 3d**

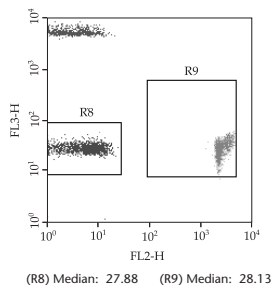
Run Cytometer Setup Beads tube C to adjust the compensation settings for FL1 – %FL2 and FL3 – %FL2.

Adjust gate R7 so that the FL2 bright bead population is located in gate R7 (*Figure 3e*). Using the FL1 – %FL2 control, adjust the median of R7 to equal the median of R6 (*Figure 3e*).



**Figure 3e**

Adjust gate R9 so that the FL2 bright bead population is located in gate R9 (*Figure 3f*). Using the FL3 – %FL2 control, adjust the median of R9 to equal the median of R8 (*Figure 3f*).



**Figure 3f**

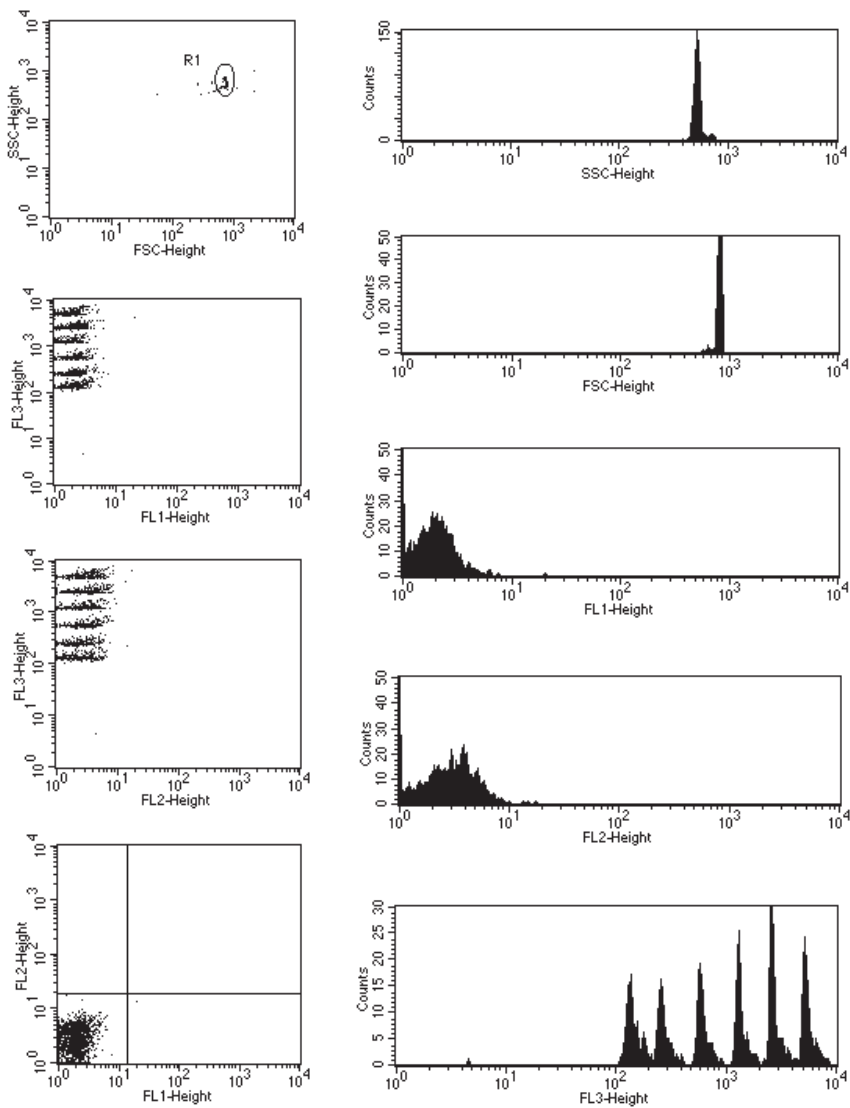
Set the FL2 – %FL3 to 0.1 if necessary. Save and print the optimized instrument settings.

## 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/pharming/CBA/downloads.shtml](http://www.bdbiosciences.com/pharming/CBA/downloads.shtml)
2. Set acquisition mode and retrieve the optimized instrument settings from *Instrument Setup with the Cytometer Setup Beads*.
3. In the Acquisition and Storage window, set the resolution to 1024.
4. Set number of events to be counted at 1800 of R1 gated events. (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 3a*).
7. Samples are now ready to be 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. Run the tubes in the order listed in *Table 2*, page 16.

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 4.** Acquisition Template Example

## 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.

1. Transfer the FACS file data for the experiment to the computer with the BD CBA Software.
2. Create two new file folders and label one "Standards" and the other "Samples".
3. Move data files to the appropriate folders.

*Note:* Only the files for control assay tubes no. 1 – 10 (the PE Detection Reagent alone and the dilution of standards) should be moved to the "Standards" file folder. All other samples should be moved to the "Samples" file folder.

Follow the instructions for analysis given in the *BD CBA Software User's Guide*.

*Note:* When entering analyte concentrations for the standards used in the experiment, it is necessary to give names to each analyte. For the Human Inflammation CBA, analyte 1 is IL-12p70, analyte 2 is TNF, analyte 3 is IL-10, analyte 4 is IL-6, analyte 5 is IL-1 $\beta$ , and analyte 6 is IL-8.

## Typical Data

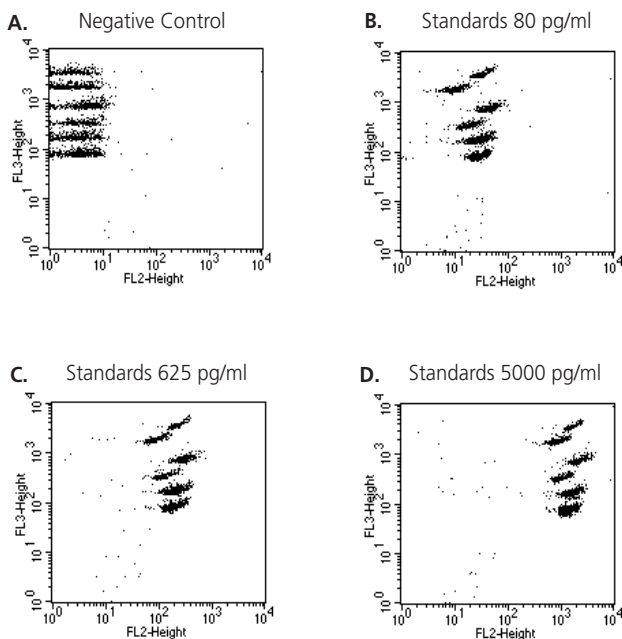
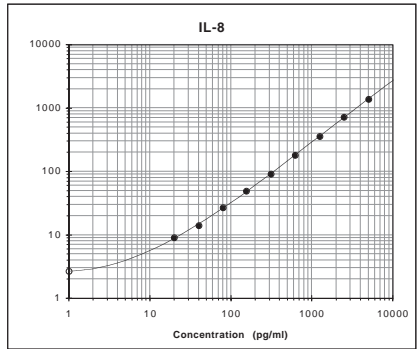
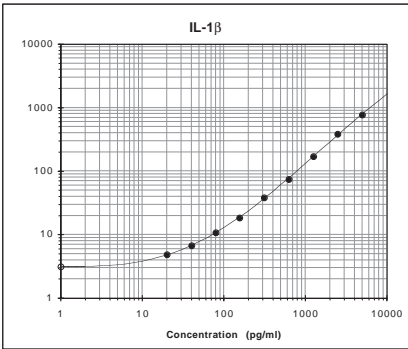
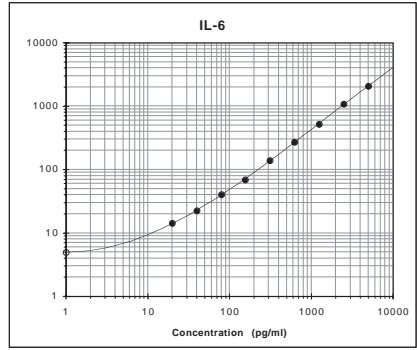
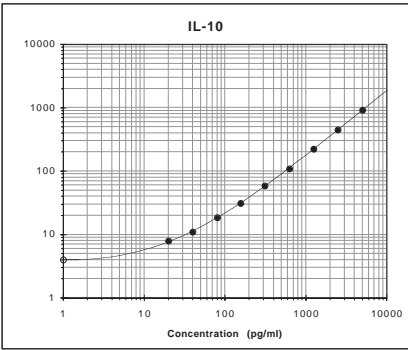
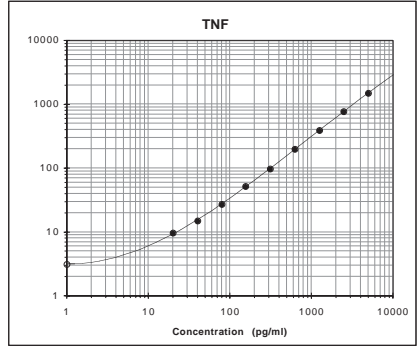
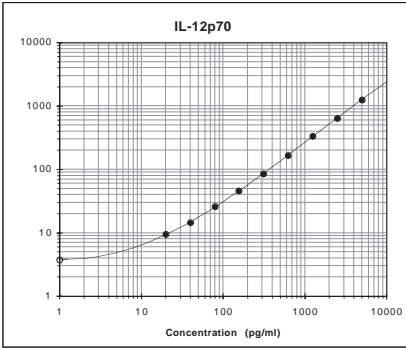


Figure 5. BD CellQuest™ Data Examples for Standards and Detectors Alone



**Figure 6. BD CBA Software Example of Standard Curves**

	Filename	SampleID	Acq Date	Dilut Factor	IL-12p70			TNF		
					FL2 MFI	Tube pg/ml	Sample pg/ml	FL2 MFI	Tube pg/ml	Sample pg/ml
1	AC090701.122	Donor700 plasma	13-Jul-01	1	4.3	4.1	4.1	2.9	<=0	
2	AC090701.123	Donor700 1h LPS+	13-Jul-01	1	4.1	3.5	3.5	939.0	3161.1	3161.1
3	AC090701.124	Donor700 1h LPS+, 1/5	13-Jul-01	5	3.9	2.5	12.6	187.7	606.0	3030.0
4	AC090701.125	Donor700 15h LPS+	13-Jul-01	1	1407.5	> 5000		7365.3	> 5000	
5	AC090701.126	Donor700 15h LPS+, 1/5	13-Jul-01	5	281.3	1047.1	5235.4	2350.1	> 5000	
6	AC090701.127	Donor700 15h LPS+, 1/10	13-Jul-01	10	127.5	463.1	4631.1	1099.0	3725.8	37258.1
7										
8										
9										
10										
11										
12										
13										
14										
15										

Figure 7. BD CBA Software Example of Sample Results

## Performance

The Human Inflammation CBA assay has been rigorously tested for performance characteristics including sensitivity, spike recovery, dilution linearity, specificity, and intra- and inter-assay precision.

Recovery and linearity results for cell-culture supernatant are using the culture supernatant assay procedure, and the serum/plasma recovery and linearity results are using the serum/plasma assay protocol.

## Sensitivity

The individual standard curve range for a given protein defines the minimum and maximum quantifiable levels using the Human Inflammation CBA (ie, 20 pg/ml and 5000 pg/ml). By applying the 4-parameter curve fit option, it is possible to extrapolate values for sample intensities not falling within the limits of the standard curve. It is up to the researcher to decide the best method for calculating values for unknown samples using this assay. The sensitivity for each protein using the Human Inflammation CBA is defined as the corresponding concentration at two standard deviations above the median fluorescence of 20 replicates of the negative control (0 pg/ml).

The sensitivity data represents the minimum performance characteristics using either the culture supernatant or serum/plasma assay procedures.

Cytokine	Median Fluorescence	Standard Deviation	Assay Sensitivity (pg/ml)
IL-8	3.4	0.4	3.6 pg/ml
IL-1 $\beta$	3.7	0.3	7.2 pg/ml
IL-6	4.7	0.4	2.5 pg/ml
IL-10	4.1	0.4	3.3 pg/ml
TNF	3.9	0.3	3.7 pg/ml
IL-12p70	4.0	0.3	1.9 pg/ml



*Note:* The assay sensitivity value shown for IL-1 $\beta$  is the result obtained at BD Biosciences. It is possible that, due to operator variation, instrument settings, and instrument performance, the investigator may not achieve the same sensitivity results.

## Recovery

Individual protein was spiked into various matrices at three different levels within the assay range. The matrices used in these experiments were not diluted before addition of the protein. The plasma samples in these experiments were EDTA treated. Results are compared with the same concentrations of the proteins spiked in the Standard Diluent, as follows:

Cytokine	Matrix	Standard spike concentration (pg/ml)	Observed in given matrix (pg/ml)	% Recovery
IL-8	Pooled Donor Sera (n = 5)	2500	2145	86%
		625	548	88%
		80	66	82%
IL-8	Pooled Donor Plasma (n = 5)	2500	2051	82%
		625	473	76%
		80	53	67%
IL-8	Cell culture supernatant	2500	2709	108%
		625	661	106%
		80	91	113%
IL-1 $\beta$	Pooled Donor Sera (n = 5)	2500	1656	66%
		625	424	68%
		80	42	52%
IL-1 $\beta$	Pooled Donor Plasma (n = 5)	2500	1776	71%
		625	403	65%
		80	47	58%
IL-1 $\beta$	Cell culture supernatant	2500	2813	113%
		625	623	100%
		80	81	101%
IL-6	Pooled Donor Sera (n = 5)	2500	1713	69%
		625	484	78%
		80	55	69%
IL-6	Pooled Donor Plasma (n = 5)	2500	2254	90%
		625	573	92%
		80	73	92%
IL-6	Cell culture supernatant	2500	2646	106%
		625	592	95%
		80	81	101%
IL-10	Pooled Donor Sera (n = 5)	2500	2016	81%
		625	556	89%
		80	67	84%
IL-10	Pooled Donor Plasma (n = 5)	2500	2308	92%
		625	589	94%
		80	70	88%
IL-10	Cell culture supernatant	2500	2449	98%
		625	600	96%
		80	79	99%
TNF	Pooled Donor Sera (n = 5)	2500	1507	60%
		625	419	67%
		80	45	56%
TNF	Pooled Donor Plasma (n = 5)	2500	1851	74%
		625	457	73%
		80	55	69%
TNF	Cell culture supernatant	2500	2864	115%
		625	665	107%
		80	88	110%
IL-12p70	Pooled Donor Sera (n = 5)	2500	1366	55%
		625	368	59%
		80	46	57%
IL-12p70	Pooled Donor Plasma (n = 5)	2500	1752	70%
		625	409	65%
		80	53	66%
IL-12p70	Cell culture supernatant	2500	2540	102%
		625	600	96%
		80	81	101%

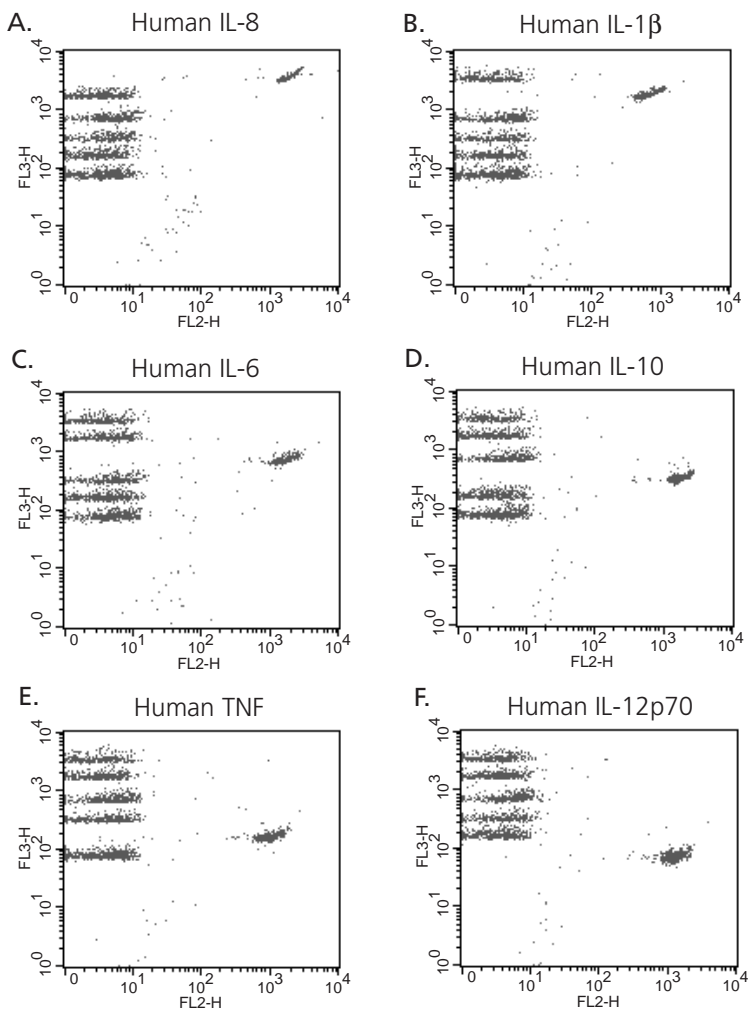
## Linearity

In two experiments, the following matrices were spiked with IL-8, IL-1 $\beta$ , IL-6, IL-10, TNF, and IL-12p70 and were then serially diluted with Assay Diluent.

Matrix	Dilution	Observed IL-8 (pg/ml)	Observed IL-1 $\beta$ (pg/ml)	Observed IL-6 (pg/ml)
Pooled Donor Sera (n = 5)	Neat	4468	2884	3410
	1:2	2583	1815	2016
	1:4	1363	900	1017
	1:8	618	458	486
	1:16	322	248	278
	1:32	129	102	99
	1:64	76	61	69
	1:128	39	46	32
	1:256	19	26	14
	Slope	1.00	0.88	1.00
	Pooled Donor Plasma (n = 5)	Neat	4493	3692
1:2		2582	2049	2654
1:4		1405	1130	1301
1:8		697	572	651
1:16		347	275	315
1:32		169	138	160
1:64		80	72	81
1:128		43	37	43
1:256		22	23	19
Slope		0.98	0.95	1.00
Cell Culture Medium		Neat	5200	5800
	1:2	2520	2665	2590
	1:4	1260	1291	1280
	1:8	602	596	635
	1:16	296	312	310
	1:32	154	143	150
	1:64	74	74	75
	1:128	39	39	41
	1:256	19	17	20
	Slope	1.01	1.04	1.01
	Matrix	Dilution	Observed IL-10 (pg/ml)	Observed TNF (pg/ml)
Pooled Donor Sera (n = 5)	Neat	4662	3213	2962
	1:2	2452	2038	1757
	1:4	1204	1041	963
	1:8	559	519	470
	1:16	304	302	252
	1:32	129	124	109
	1:64	79	83	67
	1:128	36	35	30
	1:256	18	17	15
	Slope	1.01	0.95	0.97
	Pooled Donor Plasma (n = 5)	Neat	5408	4284
1:2		4284	2270	2359
1:4		2270	1223	1218
1:8		1223	648	643
1:16		648	294	309
1:32		294	166	165
1:64		166	84	83
1:128		84	44	42
1:256		44	20	21
Slope		0.91	0.96	0.97
Cell Culture Medium		Neat	5100	6000
	1:2	2494	2597	2560
	1:4	1302	1241	1283
	1:8	643	606	618
	1:16	329	320	302
	1:32	156	157	154
	1:64	74	78	72
	1:128	40	45	42
	1:256	19	23	20
	Slope	1.01	0.99	1.02

## Specificity

The antibody pairs used in the Human Inflammation CBA assay have been screened for specific reactivity with their specific proteins. Analysis of samples containing only a single recombinant protein found no cross-reactivity or background detection of protein in other Capture Bead populations using this assay.



**Figure 8.** BD CellQuest™ Data for Detection of Individual Cytokines

## Precision

The precision results shown for intra- and inter-assay reproducibility were generated using the serum/plasma assay procedure. Most CV values are slightly improved when using the culture supernatant assay procedure due to reduced sample handling.

**Intra-assay:** Ten replicates of each of three different levels of IL-8, IL-1 $\beta$ , IL-6, IL-10, TNF, and IL-12p70 were tested.

Cytokine	IL-8			IL-1 $\beta$		
Actual Mean Conc. (pg/ml):	74	578	2221	69	540	2220
SD	3	12	104	5	19	129
% CV	4%	2%	5%	7%	4%	6%

Cytokine	IL-6			IL-10		
Actual Mean Conc. (pg/ml):	77	557	2171	76	551	2116
SD	5	25	163	5	25	121
% CV	6%	5%	8%	6%	5%	6%

Cytokine	TNF			IL-12p70		
Actual Mean Conc. (pg/ml):	70	527	1971	74	562	2094
SD	6	34	207	3	19	132
% CV	9%	6%	10%	4%	3%	6%

**Inter-assay:** Three different levels of IL-8, IL-1 $\beta$ , IL-6, IL-10, TNF, and IL-12p70 (80, 625 and 2500 pg/ml) were tested in four experiments conducted by different operators.

Cytokine	IL-8			IL-1 $\beta$		
Number of Replicates:	8	8	8	8	8	8
Actual Mean Conc. (pg/ml):	74	626	2618	69	589	2594
SD	3	24	172	9	55	196
% CV	4%	4%	7%	13%	9%	8%

Cytokine	IL-6			IL-10		
Number of Replicates:	8	8	8	8	8	8
Actual Mean Conc. (pg/ml):	78	630	2669	77	644	2669
SD	6	55	271	6	57	306
% CV	8%	9%	10%	8%	9%	11%

Cytokine	TNF			IL-12p70		
Number of Replicates:	8	8	8	8	8	8
Actual Mean Conc. (pg/ml):	74	631	2607	77	654	2563
SD	6	94	351	5	49	223
% CV	8%	15%	13%	6%	7%	9%

*Note:* The number of replicates refers to the total number of assay tubes tested at a given concentration of protein.

## Troubleshooting Tips

<b>Problem</b>	<b>Suggested Solution</b>
<b>Variation between duplicate samples.</b>	Vortex Capture Beads before pipetting. Beads can aggregate.
<b>Low bead number in samples.</b>	Avoid aspiration of beads during wash step. Do not wash or resuspend beads in volumes higher than recommended volumes.
<b>High background.</b>	Test various sample dilutions, the sample may be too concentrated. Remove excess Human Inflammation PE Detection Reagent by increasing the number of wash steps as the background may be due to non-specific binding.
<b>Little or no detection of protein in sample.</b>	Sample may be too dilute. Try various sample dilutions.
<b>Less than six bead populations are observed during analysis or distribution is unequal.</b>	Ensure that equal volumes of beads were added to each assay tube. Vortex Capture Bead vials before taking aliquots. Once Capture Beads are mixed, vortex to ensure that the beads are distributed evenly throughout the solution.
<b>Debris (FSC/SSC) during sample acquisition. Also for plasma samples.</b>	Increase FSC threshold or further dilute samples. Increase number of wash steps if necessary. Make a tighter FSC/SSC region gate around the bead population.
<b>Overlap of bead population fluorescence (FL3) during acquisition.</b>	This may occur in samples with very high protein concentration. Ensure that instrument settings have been optimized using the Cytometer Setup Beads.
<b>Standards assay tubes show low fluorescence or poor standard curve.</b>	Check that all components are properly prepared and stored. Use a new vial of standard with each experiment and once reconstituted, do not use after 12 hours. <b>Ensure that incubation times were of proper length.</b>
<b>All samples are positive or above the high standard mean fluorescence value.</b>	Dilute the samples further. The samples may be too concentrated.
<b>Biohazardous samples.</b>	It is possible to treat samples briefly with 1% paraformaldehyde before analyzing on the flow cytometer. However, this may affect assay performance and should be validated by the user.

*Note:* For best performance, vortex samples immediately before analyzing on a flow cytometer.

*Note:* The Human Inflammation CBA assay has been shown to detect IL-8, IL-6, and TNF, produced by the activation of cells from the non-human primate rhesus and cynomolgus models. Direct quantitation of proteins from the rhesus and cynomolgus models has not been validated using this kit and results may vary.

## References

1. Bishop, J.E. and K.A. Davis. 1997. A flow cytometric immunoassay for  $\beta$ 2-microglobulin in whole blood. *J. Immunol. Methods* 210:79-87.
2. Camilla C., J.P. Defoort, M. Delaage, R. Auer, J. Quintana, T. Lary, R. Hamelik, S. Prato, B. Casano, M. Martin and V. Fert. 1998. A new flow cytometry-based multi-assay system. 1. Application to cytokine immunoassays. *Cytometry Suppl.* 8:132.
3. Carson, R., and D. Vignali. 1999. Simultaneous quantitation of fifteen cytokines using a multiplexed flow cytometric assay. *J. Immunol. Methods* 227: 41-52.
4. Chen, R., L. Lowe, J.D. Wilson, E. Crowther, K. Tzeggai, J.E. Bishop and R. Varro. 1999. Simultaneous quantification of six human cytokines in a single sample using microparticle-based flow cytometric technology. *Clin. Chem.* 9:1693-1694.
5. Collins, D. P., B.J. Luebering and D.M. Shaut. 1998. T-lymphocyte functionality assessed by analysis of cytokine receptor expression, intracellular cytokine expression, and femtomolar detection of cytokine secretion by quantitative flow cytometry. *Cytometry* 33:249-55.
6. Fulton, R., R. McDade, P. Smith, L. Kienker, J. Kettman, Jr. 1997. Advanced multiplexed analysis with the FlowMetrix system. *Clin. Chem.* 43: 1749 -1756.
7. Kricka, L.J. 1996. Simultaneous multianalyte immunoassays. In *Immunoassay*. Diamandis, E.P. and T.K. Christopoulos, eds. Academic Press. pp.389-404.
8. Lund-Johansen, F., K. Davis, J. Bishop and R. de W. Malefyt. 2000. Flow cytometric analysis of immunoprecipitates: High-throughput analysis of protein phosphorylation and protein-protein interactions. *Cytometry* 39:250-259.
9. McHugh, T.M. 1994. Flow microsphere immunoassay for the quantitative and simultaneous detection of multiple soluble analytes. *Methods in Cell Biology* 42:575-595.
10. Oliver, K.G., J.R. Kettman and R.J. Fulton. 1998. Multiplexed analysis of human cytokines by use of the FlowMetrix system. *Clin. Chem.* 44:2057-60.
11. Stall, A., Q. Sun, R. Varro, L. Lowe, E. Crowther, B. Abrams, J. Bishop, and K. Davis. 1998. A single tube flow cytometric multibead assay for isotyping mouse monoclonal antibodies. Abstract LB77. Experimental Biology Meeting 1998 (late-breaking abstracts).
12. Cook, E.B., J.L. Stahl, L. Lowe, R. Chen, E. Morgan, J. Wilson, R. Varro, A. Chan, F.M. Graziano, and N.P. Barney. 2001. Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry: allergics vs. non-allergics. *J. Immunol. Methods* 254: 109-118.
13. Dotti, G., B. Salvoldo, S. Takahashi, T. Goltsova, M. Brown, D. Rill, C. Rooney, and M. Brenner. 2001. Adenovector-induced expression of human-CD40-ligand (hCD40L) by multiple myeloma cells: A model for immunotherapy. *Exp. Hematol.* 29: 952 - 961.

## Acknowledgements

The development, presentation and support of this Kit is a collaborative effort of two segments of BD Biosciences: BD Biosciences Immunocytometry Systems, San Jose, CA, and BD Biosciences Pharmingen, San Diego, CA.



**United States**

877.232.8995

**Canada**

888.259.0187

**Europe**

32.53.720.211

**Japan**

0120.8555.90

**Asia/Pacific**

65.6861.0633

**Latin America/Caribbean**

55.11.5185.9995



**BD**

**BD Biosciences**

**BD Biosciences Pharmingen**

10975 Torreyana Road

San Diego, CA 92121

Customer/Technical Service

Tel 877.232.8995 (US)

Fax 858.812.8888

**[www.bdbiosciences.com](http://www.bdbiosciences.com)**