BD™ Cytometric Bead Array (CBA)

Mouse Inflammation CBA

Kit Manual

Cat. No. 552364

Kit Contents

(Store the following items at 4°C)

A1 Mouse IL-6 Capture Beads: 1 vial, 0.8 ml

A2 Mouse IL-10 Capture Beads: 1 vial, 0.8 ml

A3 Mouse MCP-1 Capture Beads: 1 vial, 0.8 ml

A4 Mouse IFN-γ Capture Beads: 1 vial, 0.8 ml

A5 Mouse TNF-α Capture Beads: 1 vial, 0.8 ml

A6 Mouse IL-12p70 Capture Beads: 1 vial, 0.8 ml

B Mouse Inflammation PE* Detection Reagent: 1 vial, 4 ml

C Mouse 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: 1 bottle, 130 ml

G Assay Diluent: 1 bottle, 30 ml

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

Table of Contents

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 fluorescence 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 Mouse Inflammation CBA Kit can be used to quantitatively measure Interleukin-6 (IL-6), Interleukin-10 (IL-10), Monocyte Chemoattractant Protein-1 (MCP-1), Interferon-γ (IFN-γ), Tumor Necrosis Factor-α (TNF-α), 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.

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-6, IL-10, MCP-1, IFN-γ, TNF-α, 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.

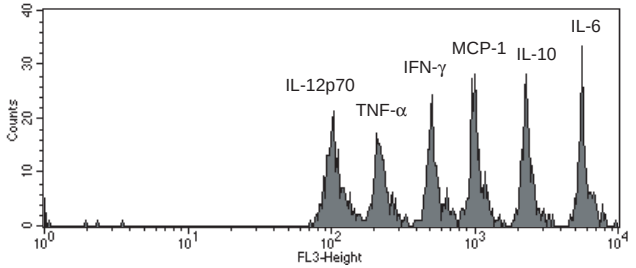


Figure 1

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 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 Mouse 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. Note the reduced sensitivity of the Mouse MCP-1 assay *(see sensitivity and precision information on pgs. 21 and 25)*.

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 Immuncytometry Systems, San Jose, CA) flow cytometers.

Serum spike recoveries for IL-10 and TNF-α are lower than for the other proteins in this assay. This variation is due to assay conditions and serum proteins. It may affect quantitation of these proteins in serum samples.

Reagents Provided

Bead Reagents

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

Bead	Specificity
(Brightest) A1	IL-6
A2	IL-10
A3	MCP-1
A4	IFN-γ
A5	TNF-α
(Dimmest) A6	IL-12p70

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 µl/test.

Antibody and Standard Reagents

Mouse Inflammation PE Detection Reagent (B): A 75-test vial of PE-conjugated anti-mouse IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 antibodies that is formulated for use at 50 µl/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 µl/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 µl/test. This reagent is used with the Cytometer Setup Beads to set the initial instrument compensation settings. Store at 4°C. Do not freeze.

Mouse Inflammation Standards (C): Two vials containing lyophilized recombinant mouse proteins. Each vial should be reconstituted in 0.2 ml of Assay Diluent to prepare a 10× bulk standard. The reconstituted 10× bulk standard contains 50 ng/ml of each recombinant mouse IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 protein. Store at 4°C.

Note: The Mouse Inflammation Standards vials are stable until the kit expiration date. Following reconstitution, store the freshly reconstituted 10× 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×) used to reconstitute and dilute the Mouse Inflammation Standards and to dilute test samples. Store at 4°C.

Wash Buffer (F): A single 130 ml bottle of phosphate buffered saline (PBS) solution (1×), 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.

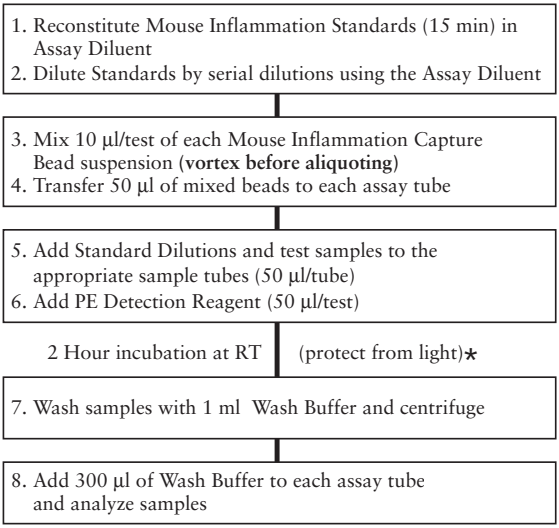
Materials Required but not Provided

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

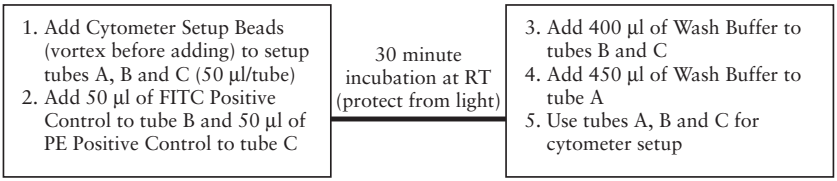
- A flow cytometer equipped with a 488-nm laser capable of detecting and distinguishing fluorescence emissions at 576 and 670 nm (eg, BD FACScan or BD FACSCalibur instruments) and BD CellQuest™ Software.
- 12 × 75 mm sample acquisition tubes for a flow cytometer (eg, BD Falcon™ Cat. No. 352008.)
- BD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



***Cytometer Setup Bead Procedure**



Preparation of Mouse Inflammation Standards

The Mouse 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 Mouse 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 µl of Assay Diluent to each of the remaining tubes.
5. Transfer 100 µl of 10× 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 ***(see Figure 2)***. The Assay Diluent serves as the negative control.

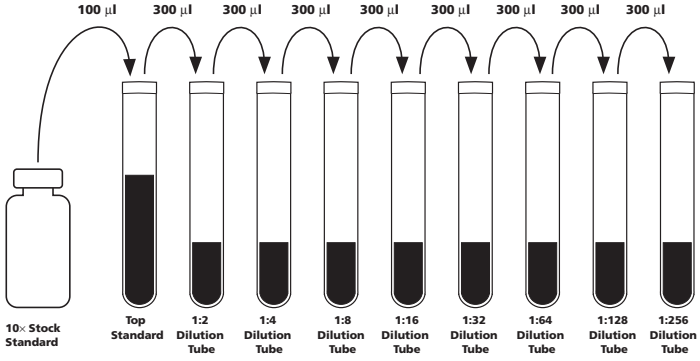


Figure 2. Preparation of Mouse Inflammation Standard Dilutions

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

Table 1. Mouse 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
Mouse IL-6	5000	2500	1250	625	312.5	156	80	40	20
Mouse IL-10	5000	2500	1250	625	312.5	156	80	40	20
Mouse MCP-1	5000	2500	1250	625	312.5	156	80	40	20
Mouse IFN-γ	5000	2500	1250	625	312.5	156	80	40	20
Mouse TNF-α	5000	2500	1250	625	312.5	156	80	40	20
Mouse IL-12p70	5000	2500	1250	625	312.5	156	80	40	20

Preparation of Mixed Mouse 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 µl aliquot of each Capture Bead, for each assay tube to be analyzed, into a single tube labeled "mixed Capture beads" (eg, 10 µl of IL-6 Capture Beads × 18 assay tubes = 180 µl of IL-6 Capture Beads required).
4. Vortex the Bead mixture thoroughly.

The mixed Capture beads are now ready to be transferred to the assay tubes (50 µl of mixed Capture beads/tube) as described in Mouse Inflammation CBA Assay Procedure, pg. 12.

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.

Mouse Inflammation CBA 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. **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 in Cytometer Setup, Data Acquisition, and Analysis on pg. 13.

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 Mouse Inflammation Standard dilutions to the control assay tubes.
3. Add 50 µl of each test sample to the test assay tubes.
4. Add 50 µl of the Mouse Inflammation PE Detection Reagent to the assay tubes.
5. Incubate the assay tubes for 2 hours at RT and protect from direct exposure to light. During this incubation, perform the Cytometer Setup procedure described in Cytometer Setup, Data Acquisition, and Analysis on pgs. 13 - 14.
6. Add 1 ml of Wash Buffer to each assay tube and centrifuge at 200 × *g* for 5 minutes.
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 seconds immediately before analyzing on the flow cytometer.**

Table 2. Essential control assay tubes

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

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

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 FACStation CD for Macintosh computers in the BD CBA folder. Following installation on Macintosh computers using BD CBA Software Version 1.0, the template can be found in the BD Applications/BD CBA folder/Sample Files/Mouse Isotyping Files/Instrument Setup folder. For BD CBA Software Version 1.1 or higher, the template can be found in the BD Applications/BD CBA folder. The template is not installed from the CD on PC-compatible computers. This file and instrument set-up templates for dual-laser and other flow cytometers may also be downloaded via the internet from: 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 pgs. 15 - 16.

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)***.

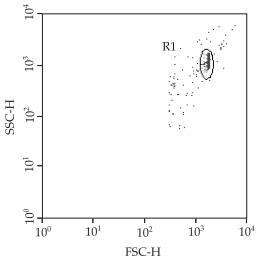


Figure 3a

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.

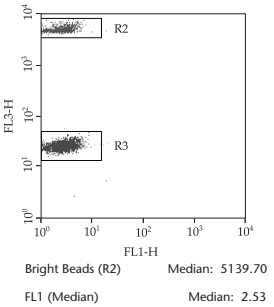


Figure 3b

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)*.**

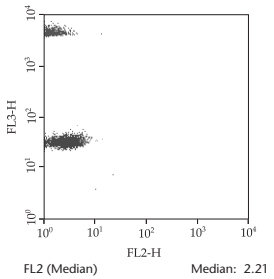


Figure 3c

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)***.

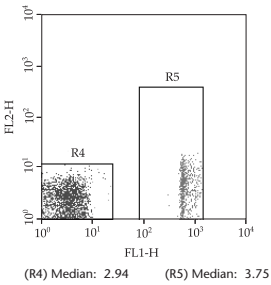


Figure 3d

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)***.

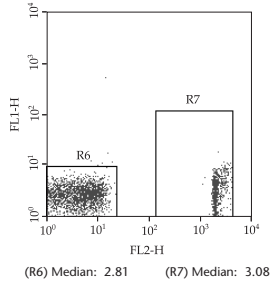


Figure 3e

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)***.

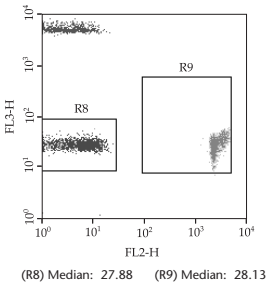


Figure 3f

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 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. It is labeled "Isotype Kit Acquire Template". Alternatively, the Acquisition template may be downloaded via the internet from: http://www.bdbiosciences.com/pharmingen/CBA/downloads.shtml

2. Set acquisition mode and retrieve the optimized instrument settings from Instrument Setup with the Cytometer Setup Beads on pg. 14.

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 of Mouse Inflammation CBA Assay Procedure on pg. 12.

   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).

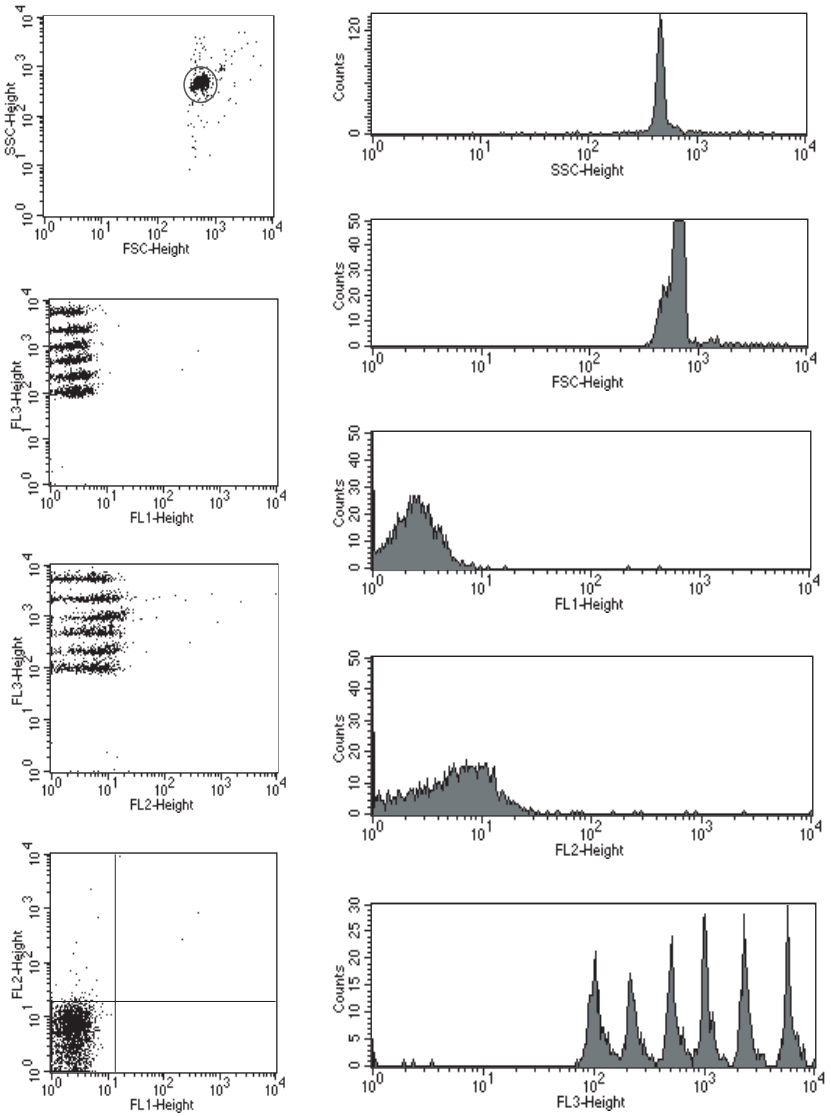


Figure 4. Acquisition Template Example

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 Mouse Inflammation CBA, analyte 1 is IL-12p70, analyte 2 is TNF-α, analyte 3 is IFN-γ, analyte 4 is MCP-1, analyte 5 is IL-10, and analyte 6 is IL-6.**

Typical Data

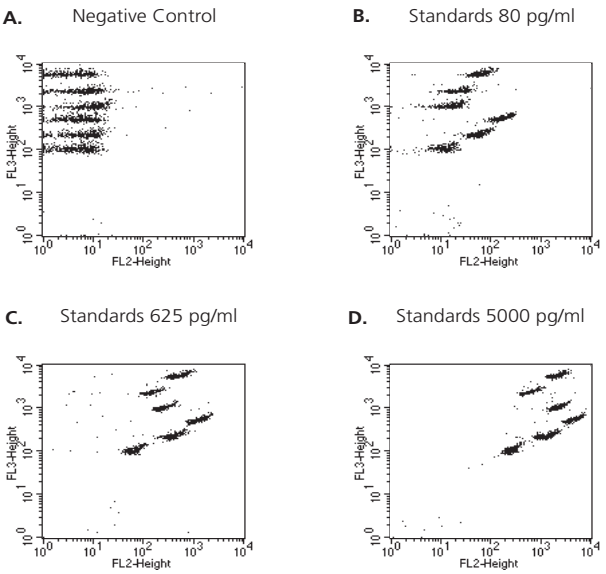


Figure 5. BD CellQuest Data Examples for Standards and Detectors Alone

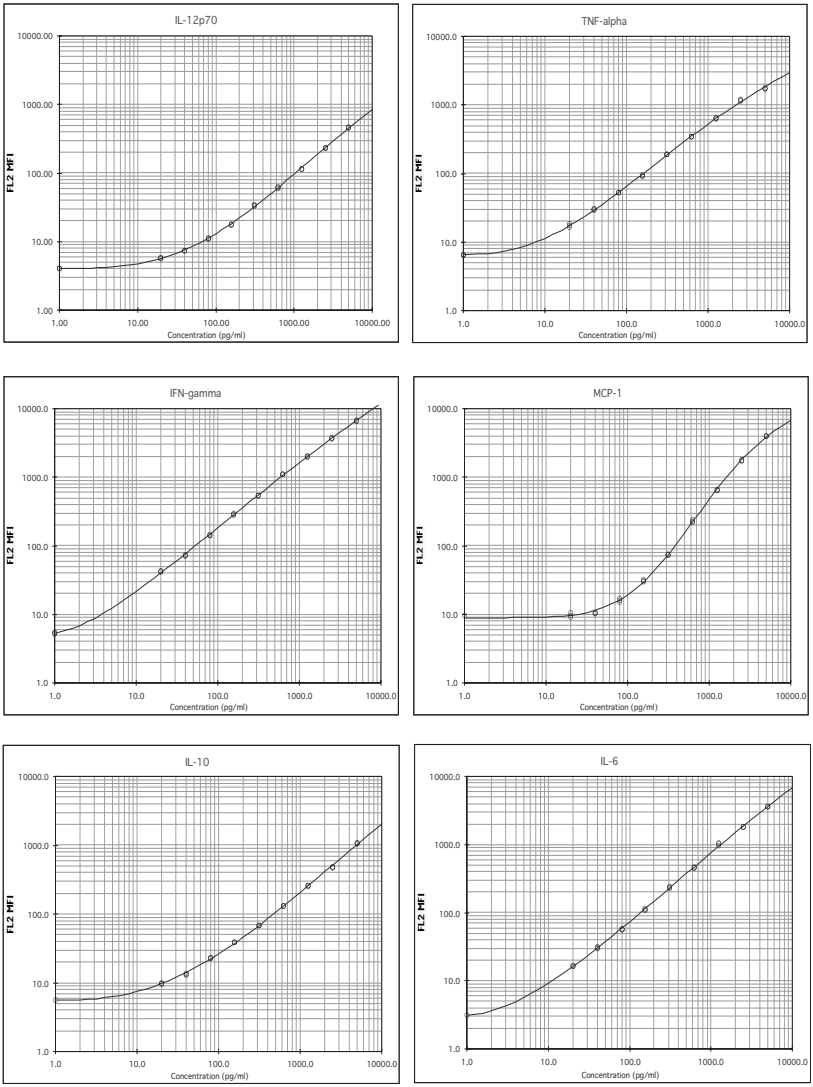


Figure 6. BD CBA Software Example of Standard Curves

BD Cytometric Bead Array Analysis

					IL-12p70			TNF-alpha		
	Filename	SampleID	Acq Date	Dilut Factor	FL2 MFI	Tube pg/ml	Sample pg/ml	FL2 MFI	Tube pg/ml	Sample pg/ml
1	072302BB.045	Mouse sup neat	23-Jul-02	1	266.6	2855.2	2855.2	2195.1	> 5000	
2	072302BB.046	Mouse sup 1;2	23-Jul-02	2	144.6	1518.3	3036.7	2184.3	> 5000	
3	072302BB.047	Mouse sup 1;4	23-Jul-02	4	68.8	708.5	2834.1	2148.0	> 5000	
4	072302BB.048	Mouse sup 1;16	23-Jul-02	16	19.5	172.2	2755.7	1998.9	> 5000	
5	072302BB.049	Mouse sup 1;64	23-Jul-02	64	8.0	45.1	2889.3	805.8	1674.5	107170.6
6	072302BB.050	Mouse sup 1;256	23-Jul-02	256	5.0	13.4	3427.4	225.7	386.6	98974.6
7	072302BB.051	Mouse sup 1;1024	23-Jul-02	1024	5.7	19.9	20423.2	59.4	90.9	93116.3
8										
9										
10										
11										

Figure 7. BD CBA Software Example of Sample Results

Performance

The Mouse 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 Mouse 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 Mouse 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.

Protein	Median Fluorescence	Standard Deviation	Assay Sensitivity (pg/ml)
IL-6	3.3	0.3	5
IL-10	5.9	0.5	17.5
MCP-1	9.3	0.4	52.7
IFN-γ	5.4	0.4	2.5
TNF-α	6.5	0.3	7.3
IL-12p70	4.1	0.4	10.7

Recovery

Individual protein was spiked into various matrices at three different levels within the assay range. The cell culture media used in these experiments were not diluted before addition of the protein. The pooled mouse serum samples in these experiments were diluted 1:4 in Assay Diluent before addition of the cytokine protein. Results are compared with the same concentrations of the proteins spiked in the Assay Diluent, as follows:

Protein	Matrix	Standard Spike Concentration (pg/ml)	Observed in Given Matrix (pg/ml)	% Recovery
IL-6	Pooled mouse sera 1:4 dilution	2500 625 80	1990.3 458.4 62.3	80% 73% 78%
IL-6	Cell culture media	2500 625 80	2476.7 594.3 95.8	99% 95% 120%
IL-10	Pooled mouse sera 1:4 dilution	2500 625 80	918.5 208.2 11.2	37% 33% 14%
IL-10	Cell culture media	2500 625 80	2326.7 581.9 99.7	93% 93% 125%
MCP-1	Pooled mouse sera 1:4 dilution	2500 625 80	1954.5 448.5 35.8	78% 72% 45%
MCP-1	Cell culture media	2500 625 80	2474.2 579.2 82.7	99% 93% 103%
IFN-γ	Pooled mouse sera 1:4 dilution	2500 625 80	1386.8 323.9 46.6	55% 52% 58%
IFN-γ	Cell culture media	2500 625 80	2423.9 584.8 79.7	97% 94% 100%
TNF-α	Pooled mouse sera 1:4 dilution	2500 625 80	779.8 181.0 26.8	31% 29% 34%
TNF-α	Cell culture media	2500 625 80	2651.7 587.5 83.3	106% 94% 104%
IL-12p70	Pooled mouse sera 1:4 dilution	2500 625 80	1468.5 350.5 23.5	59% 56% 29%
IL-12p70	Cell culture media	2500 625 80	2349.0 538.2 73.9	94% 86% 92%

Linearity

In two experiments, the following matrices were spiked with IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 and were then serially diluted with Assay Diluent.

Matrix	Dilution	Observed IL-6 (pg/ml)	Observed IL-10 (pg/ml)	Observed MCP-1 (pg/ml)
Pooled mouse sera 1:4 starting dilution	1:4	4041.8	1935.9	4052.3
	1:8	2128.9	1244.7	2227.9
	1:16	1086.9	797.3	1183.0
	1:32	529.3	438.6	619.2
	1:64	277.1	238.7	291.8
	1:128	136.9	136.7	160.5
	1:256	70.6	76.7	77.8
	1:512	33.2	29.9	22.3
	1:1024	14.5	14.2	0.0
	Slope	1.006	0.882	1.029
Cell culture medium	Neat	4347.7	4870.6	4946.1
	1:2	2370.3	2373.0	2379.3
	1:4	1207.6	1271.9	1202.2
	1:8	572.5	588.9	593.1
	1:16	295.0	312.8	296.4
	1:32	132.3	151.0	135.7
	1:64	68.9	72.9	57.8
	1:128	39.8	44.2	34.6
	1:256	17.5	21.6	18.1
	Slope	0.998	0.979	1.026

Matrix	Dilution	Observed IFN-γ (pg/ml)	Observed TNF-α (pg/ml)	Observed IL-12p70 (pg/ml)
Pooled mouse sera 1:4 starting dilution	1:4	2877.2	1601.9	3372.1
	1:8	1588.3	1001.2	1675.3
	1:16	958.8	630.0	913.5
	1:32	520.4	370.7	491.7
	1:64	255.4	211.5	242.8
	1:128	140.1	122.0	142.2
	1:256	69.9	73.4	82.7
	1:512	34.2	38.0	34.4
	1:1024	18.1	17.5	12.9
	Slope	0.922	0.801	0.961
Cell culture medium	Neat	4518.4	4407.9	4615.7
	1:2	2392.4	2576.8	2426.8
	1:4	1255.9	1330.1	1203.6
	1:8	640.7	616.1	588.7
	1:16	303.3	321.2	328.2
	1:32	145.3	146.3	130.6
	1:64	72.2	75.8	63.0
	1:128	38.6	43.2	44.5
	1:256	20.9	21.6	19.1
	Slope	0.999	0.979	1.113

Specificity

The antibody pairs used in the Mouse 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.

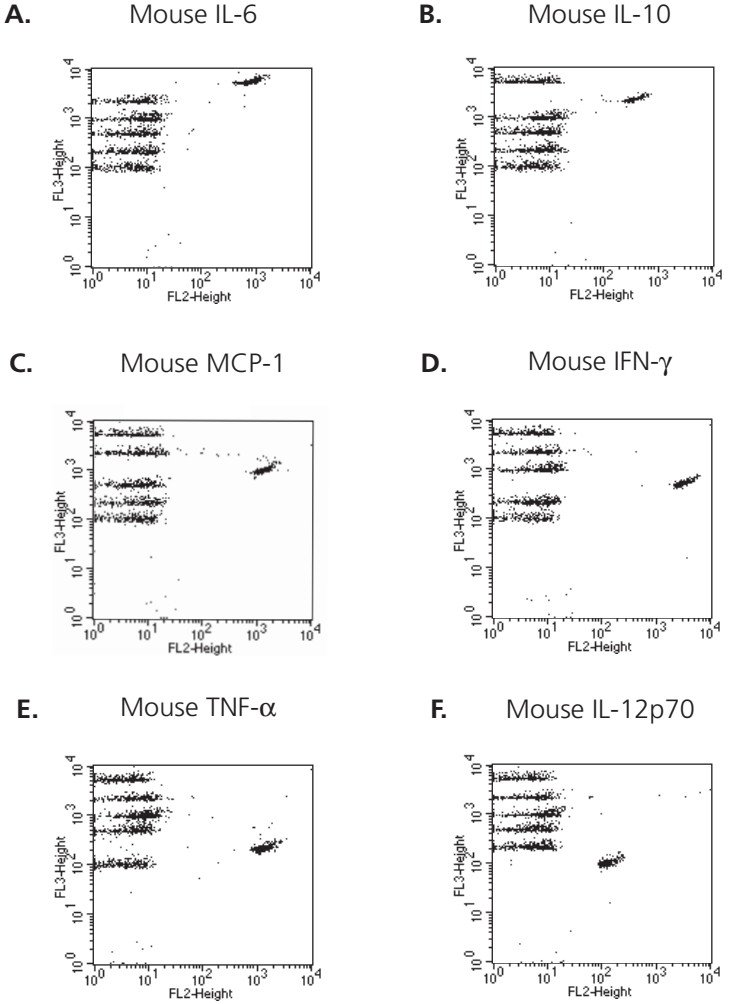


Figure 8. BD CellQuest Data for Detection of Individual Proteins

Precision

Intra-assay: Ten replicates of each of three different levels of IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 were tested.

Protein	IL-6			IL-10		
Actual Mean Conc. (pg/ml):	76	551	2107	78	573	1987
SD	4	20	94	7	19	104
% CV	5%	4%	5%	9%	3%	5%
Protein	**MCP-1**			**IFN-γ**		
Actual Mean Conc. (pg/ml):	66	614	2300	73	633	2239
SD	6	9	46	3	19	103
% CV	10%	2%	2%	4%	3%	5%
Protein	**TNF-α**			**IL-12p70**		
Actual Mean Conc. (pg/ml):	69	597	2358	66	600	2238
SD	2	21	103	10	23	103
% CV	3%	4%	4%	15%	4%	5%

Inter-assay: Three different levels of IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 (80, 625 and 2500 pg/ml) were tested in four experiments.

Protein	IL-6			IL-10		
Number of Replicates:	8	8	8	8	8	8
Actual Mean Conc. (pg/ml):	79	557	2426	71	581	2329
SD	8	62	198	10	45	162
% CV	10%	11%	8%	14%	8%	7%
Protein	**MCP-1**			**IFN-γ**		
Number of Replicates:	8	8	8	8	8	8
Actual Mean Conc. (pg/ml):	58	598	2431	75	631	2425
SD	11	33	51	6	40	120
% CV	19%	5%	2%	8%	6%	5%
Protein	**TNF-α**			**IL-12**		
Number of Replicates:	8	8	8	8	8	8
Actual Mean Conc. (pg/ml):	75	592	2697	72	590	2430
SD	8	40	155	7	30	70
% CV	11%	7%	6%	9%	5%	3%

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

Troubleshooting Tips

Problem	Suggested Solution
Variation between duplicate samples.	Vortex Capture Beads before pipetting. Beads can aggregate.
Low bead number in samples.	Avoid aspiration of beads during wash step. Do not wash or resuspend beads in volumes higher than recommended volumes.
High background.	Test various sample dilutions, the sample may be too concentrated. Remove excess Mouse Inflammation PE Detection Reagent by increasing the number of wash steps as the background may be due to non-specific binding.
Little or no detection of protein in sample.	Sample may be too dilute. Try various sample dilutions.
Less than six bead populations are observed during analysis or distribution is unequal.	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.
Debris (FSC/SSC) during sample acquisition. Also for plasma samples.	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.
Overlap of bead population fluorescence (FL3) during acquisition.	This may occur in samples with very high protein concentration. Ensure that instrument settings have been optimized using the Cytometer Setup Beads.
Standards assay tubes show low fluorescence or poor standard curve.	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. Ensure that incubation times were of proper length.
All samples are positive or above the high standard mean fluorescence value.	Dilute the samples further. The samples may be too concentrated.
Biohazardous samples.	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 Mouse Inflammation CBA assay has been shown to detect TNF-α produced by the activation of cells from the rat model. Direct quantitation of proteins from the rat model has not been validated using this kit and results may vary.

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Notes

Notes

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