

BD™ Cytometric Bead Array (CBA)

Human Chemokine Kit

Instruction Manual

Cat. No. 552990



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Kit Contents

80 Tests (50 samples and 2 standard curves)

(Store the following items at 4°C)

- A1 Human CXCL8/IL-8 Capture Beads: 1 vial, 0.8 ml
- A2 Human CCL5/RANTES Capture Beads: 1 vial, 0.8 ml
- A3 Human CXCL9/MIG Capture Beads: 1 vial, 0.8 ml
- A4 Human CCL2/MCP-1 Capture Beads: 1 vial, 0.8 ml
- A5 Human CXCL10/IP-10 Capture Beads: 1 vial, 0.8 ml
- B Human Chemokine PE Detection Reagent: 1 vial, 4 ml
- C Human Chemokine 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

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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 Kit 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 BD CBA kit 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 BD CBA assays to use fewer sample dilutions and to determine the concentration of an unknown analyte in substantially less time (compared to conventional ELISA).

The BD CBA Human Chemokine Kit can be used to quantitatively measure Interleukin-8 (CXCL8/IL-8), RANTES (CCL5/RANTES), Monokine-induced by Interferon- γ (CXCL9/MIG), Monocyte Chemoattractant Protein-1 (CCL2/MCP-1), and Interferon- γ -induced Protein-10 (CXCL10/IP-10) levels in a single sample. The Kit performance has been optimized for analysis of specific chemokines in tissue culture supernatants, EDTA-treated plasma (EDTA-plasma), and serum samples.

Principle of the Test

Five bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10. The five bead populations are mixed together to form the bead array that is resolved in a red channel (ie, FL3 or FL4) of a flow cytometer. (see *Figure 1*).

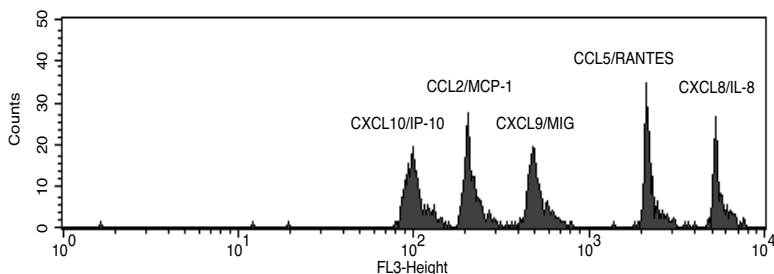


Figure 1.

The Capture Beads are mixed 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 formats using the BD CBA Analysis Software or FCAP Array™ 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-fifth the quantity necessary for conventional ELISA assays due to the detection of five analytes in a single sample.
- A single set of diluted standards is used to generate a standard curve for each analyte.
- A BD CBA experiment takes less time than a single ELISA and provides results that would normally require five conventional ELISAs.

Limitations

The Theoretical limit of detection of the BD CBA Human Chemokine Kit is comparable to conventional ELISA, but due to the complexity and kinetics of this multi-analyte assay, the actual limit of detection on a given experiment may vary slightly (see *Theoretical Limit of Detection and Precision* in the Performance section).

The BD CBA Kit 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 BD FACStar™ Plus and BD FACSVantage™ flow cytometers.

Serum and EDTA-plasma spike recoveries for CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10 are lower than for other proteins in this assay. This variation is due to assay conditions and serum or EDTA-plasma proteins and may affect quantitation of these proteins in serum or plasma samples.

When testing serum samples for CXCL10/IP-10 or CCL2/MCP-1, it is recommended that the serum sample be diluted 1:10 or 1:4 respectively. This is to reduce the effects of serum interference with the quantitation of these proteins in serum samples.

Quantitative results or protein levels for the same sample or recombinant protein run in ELISA and BD CBA assays may differ. A spike recovery assay can be performed using an ELISA standard followed by BD CBA analysis to assess possible differences in quantitation.

This kit is designed to be used as an integral unit. Do not mix components from different batches or kits.

Reagents Provided

Bead Reagents

Human Chemokine Capture Beads (A1 – A5): The specific Capture Beads, having discrete fluorescence intensity characteristics, are distributed from brightest to dimmest as follows:

Bead	Specificity
(Brightest) A1	CXCL8/IL-8
A2	CCL5/RANTES
A3	CXCL9/MIG
A4	CCL2/MCP-1
(Dimmest) A5	CXCL10/IP-10

A single, 80-test vial of each specific Capture Bead (A1 – A5) 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.

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

US Orders: 877.232.8995

Antibody and Standard Reagents

Human Chemokine PE Detection Reagent (B): An 80-test vial of PE-conjugated anti-human CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10 antibodies that is formulated for use at 50 μ l/test. Store at 4°C. Do not freeze.

Human Chemokine Standards (C): Two vials containing lyophilized recombinant human proteins. Each vial should be reconstituted in 4.0 ml of Assay Diluent to prepare the top standard. Store at 4°C.

PE Positive Control Detector (E1): A 10-test vial of PE-conjugated antibody control that is formulated for use at 50 μ 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 μ 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.

Buffer Reagents:

Wash Buffer (F): A single 130-ml bottle 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.

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

Warnings and Precautions

Hazardous Ingredients:

Sodium Azide:

Components A1 – A5, B, D, 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.

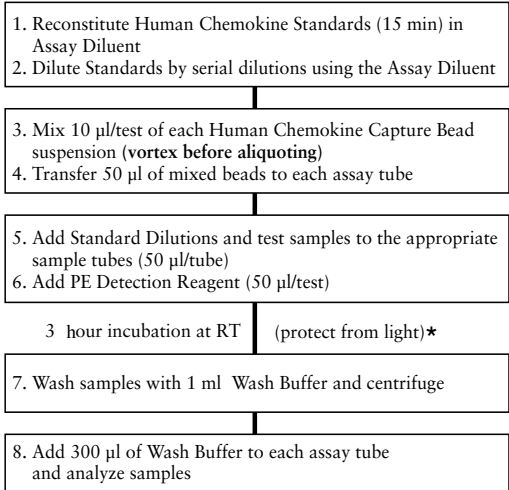
** Source of all serum proteins is from USDA inspected abattoirs located in the United States.*

Materials Required but Not Provided

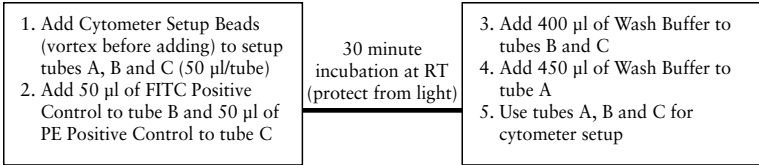
In addition to the reagents provided in the BD CBA Human Chemokine Kit, the following items are also required:

- A flow cytometer equipped with a laser capable of emitting light at 488 nm and detecting and distinguishing fluorescent 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 or FCAP Array software (Cat. No. 641488).
Note: The BD CBA Software is no longer available for purchase but is still supported on existing compatible systems.
- BD Calibrite™ 3 Beads (Cat. No. 340486).

Overview: BD CBA Human Chemokine Kit Assay Procedure



*Cytometer Setup Bead Procedure



Preparation of Human Chemokine Standards

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

1. Open one vial of lyophilized Human Chemokine Standards. Transfer the standard spheres to a polypropylene tube (eg, 15 ml Conical Tube, BD Falcon Cat. No. 352097). Label tube “Top Standard”.
2. Reconstitute the standards with 4.0 ml of Assay Diluent. Allow the reconstituted standard to equilibrate for at least 15 minutes before making dilutions. **Mix reconstituted protein by pipette only. Do not vortex or mix vigorously.**
3. Label 12 × 75 mm tubes (BD Falcon Cat. No.352008) and arrange them in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128 and 1:256.
4. Pipette 300 µl of Assay Diluent to each of the remaining tubes.
5. 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*). **Mix by pipette only, do not vortex.** Prepare one tube containing Assay Diluent to serve as the 0 pg/ml negative control.

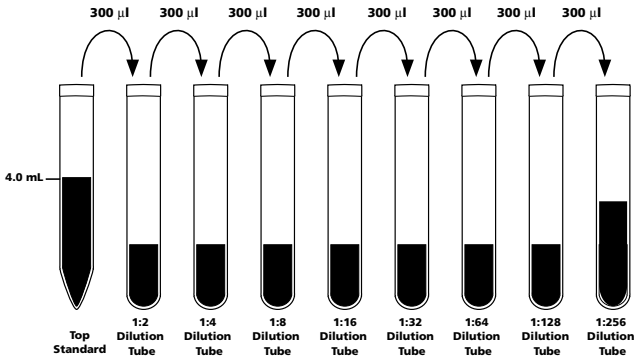


Figure 2. Preparation of Human Chemokine Standard Dilutions

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

Table 1. Human Chemokine 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 CXCL8/IL-8	2500	1250	625	312.5	156	80	40	20	10
Human CCL5/RANTES	2500	1250	625	312.5	156	80	40	20	10
Human CXCL9/MIG	2500	1250	625	312.5	156	80	40	20	10
Human CCL2/MCP-1	2500	1250	625	312.5	156	80	40	20	10
Human CXCL10/IP-10	2500	1250	625	312.5	156	80	40	20	10

Preparation of Mixed Human Chemokine Capture Beads

The Capture Beads are bottled individually, and it is necessary to pool the bead reagents (A1 – A5) 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 (eg, 8 unknowns, 9 chemokine standard dilutions, and 1 negative control = 18 assay tubes).

Note: Extra tests of Capture Beads should be mixed to ensure that the necessary number of tests will be recovered from the mixed Capture Beads tube (ie, add an additional 2 – 3 assay tubes to the number determined in Step 1 above before calculating the amount to add to the mixed Capture Beads tube in Step 3).

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 CXCL8/IL-8 Capture Beads × 18 assay tubes = 180 µl of CXCL8/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 µl of mixed Capture Beads/tube) as described in *Human Chemokine Kit Assay Procedure*.

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 10 – 2500 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 protein 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.

BD CBA Human Chemokine Kit 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 Chemokine Standards and the Cytometer Setup controls in each experiment.** See *Table 2* for a detailed description of the reagents added to the Chemokine Standard control assay tubes. The Cytometer Setup procedure is described in *Cytometer Setup, Data Acquisition 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 Chemokine 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 Human Chemokine PE Detection Reagent to the 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 in Preparation of Cytometer Setup Beads, Instrument Setup with BD FACSComp™ Software and BD Calibrite Beads, and Instrument Setup with the Cytometer Setup Beads.
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 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.**

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

Tube No.	Reagents (All reagent volumes are 50 µl)
1 (Negative Control 0 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Assay Diluent
2 (10 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Chemokine Standards 1:256 Dilution
3 (20 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Chemokine Standards 1:128 Dilution
4 (40 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Chemokine Standards 1:64 Dilution
5 (80 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Chemokine Standards 1:32 Dilution
6 (156 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Chemokine Standards 1:16 Dilution
7 (312.5 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Chemokine Standards 1:8 Dilution
8 (625 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Chemokine Standards 1:4 Dilution
9 (1250 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Chemokine Standards 1:2 Dilution
10 (2500 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Chemokine 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 or FCAP Array Software.

Additional setup protocols for the BD FACSCalibur flow cytometer (dual laser), BD FACSArray™ bioanalyzer, and other BD FACS brand flow cytometers can be found at bdbiosciences.com/cbasetup.

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 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 FACStation CD for Macintosh computers in the BD CBA folder. This file may also be downloaded via the internet from: bdbiosciences.com/cbatemplates

2. Set the instrument to Acquisition mode.

Note: The data will be evaluated in five parameters (FSC, SSC, FL1, FL2, and FL3). Turn off additional detectors.

3. Set SSC (side light scatter) and FSC (forward light scatter) to Log mode.
4. Decrease the SSC PMT voltage by 100 from what BD FACSComp set.
5. Set the Threshold to SSC at 650.
6. In setup mode, run Cytometer Setup Beads tube A. Follow the setup instructions on the following pages.

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*).

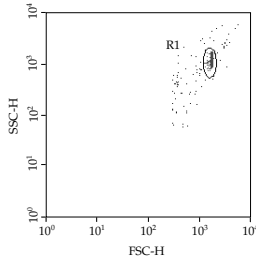


Figure 3a

Adjust the FL3 PMT so that the median intensity of the top FL3 bead population 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.

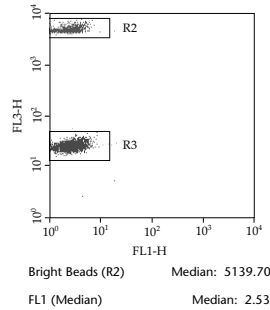


Figure 3b

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*).

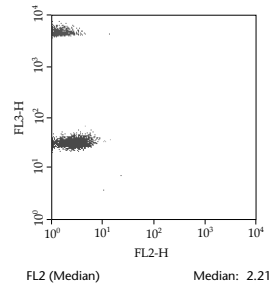


Figure 3c

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*).

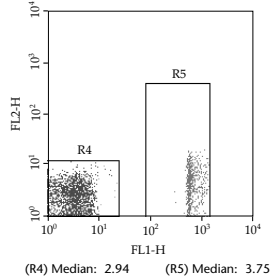


Figure 3d

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*).

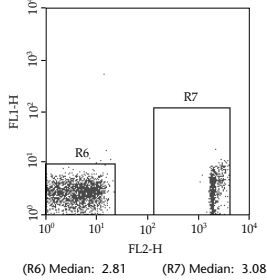


Figure 3e

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*).

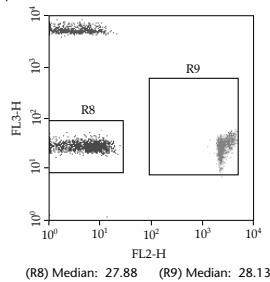


Figure 3f

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

Data Acquisition

1. Open the acquisition template.

Note: The acquisition template may be downloaded from:
bdbiosciences.com/cbatemplates

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 1500 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 Human Chemokine Kit Assay Procedure.

File names must be alphanumeric (ie, contain at least one letter).

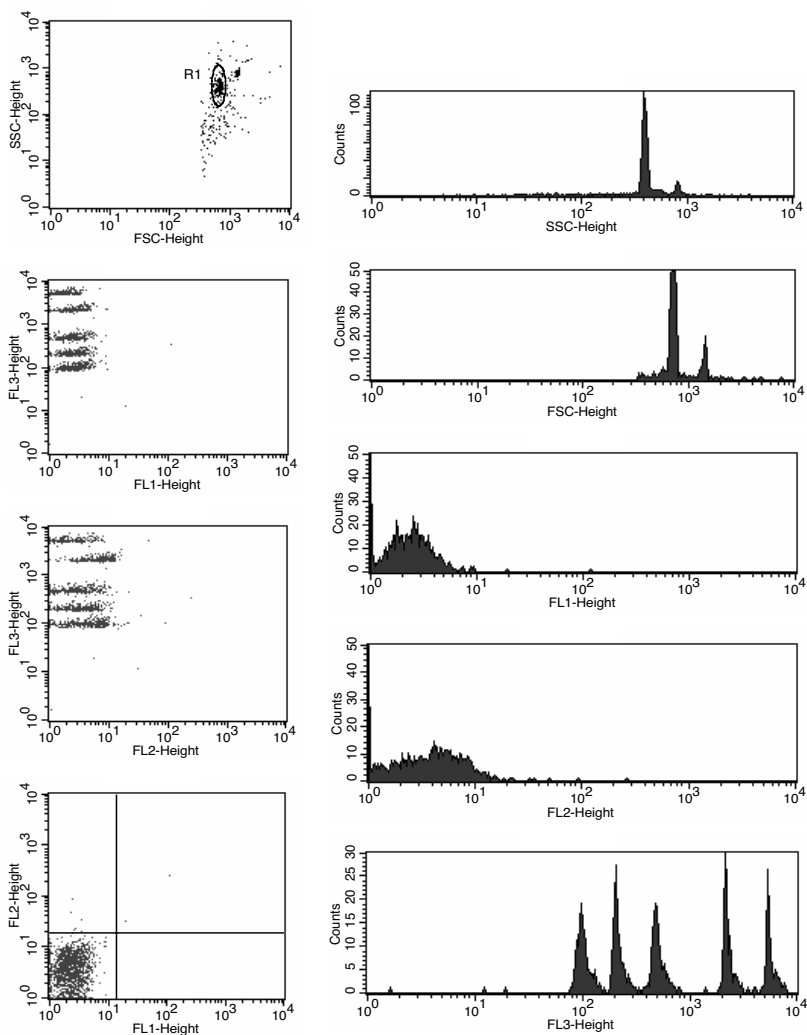


Figure 4. Acquisition Template Example

Analysis of Sample Data

The analysis of BD CBA data can be accomplished using BD CBA Software or FCAP Array software. For BD CBA Software, please refer to the User's Guide for instructions. For FCAP Array software, please visit the following link for instructions: bdbiosciences.com/kitanalysis

Typical Data

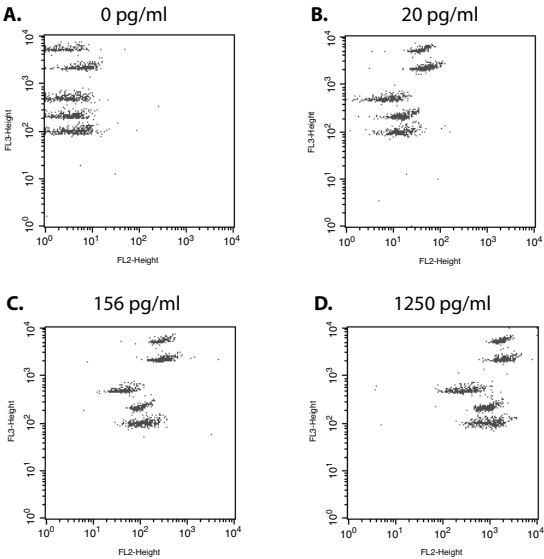


Figure 5. BD CellQuest Data Examples for Standards and Detectors Alone

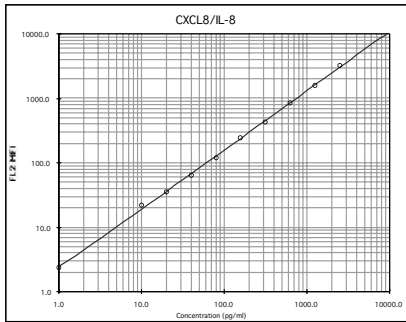
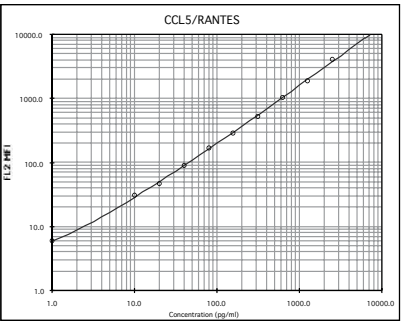
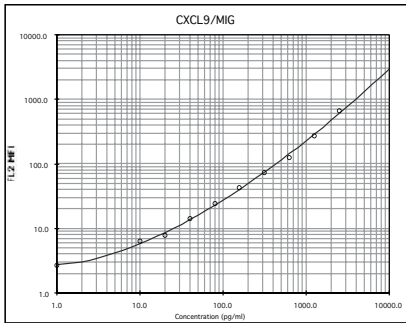
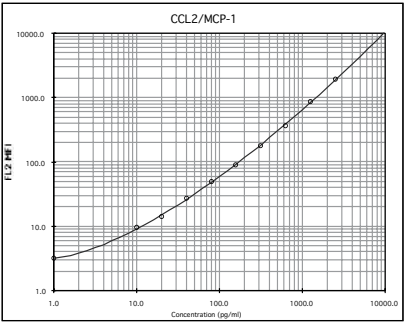
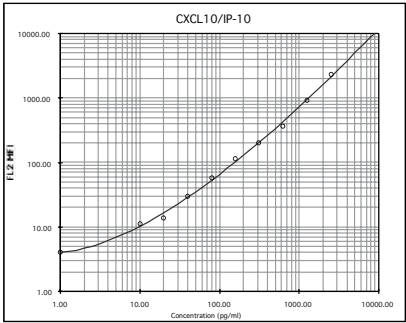


Figure 6. Example of Standard Curves

Performance

The BD CBA Human Chemokine Kit assay has been rigorously tested for the following: theoretical limit of detection, spike recovery, dilution linearity, specificity, and intra- and inter-assay precision.

Theoretical Limit of Detection

The individual standard curve range for a given protein defines the minimum and maximum quantifiable levels using the BD CBA Human Chemokine Kit (ie, 10 pg/ml and 2500 pg/ml.) By applying the 4-parameter curve fit option, it is possible to interpolate values for sample intensities below 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 theoretical limit of detection of the BD CBA Human Chemokine Kit for each protein is defined as the corresponding concentration at two standard deviations above the median fluorescence of 20 replicates of the negative control (0 pg/ml).

Protein	Median Fluorescence	Standard Deviation	Limit of Detection (pg/ml)
CXCL8/IL-8	2.6	0.2	0.2
CCL5/RANTES	5.5	0.3	1.0
CXCL9/MIG	2.9	0.2	2.5
CCL2/MCP-1	3.3	0.2	2.7
CXCL10/IP-10	4.1	0.3	2.8

Recovery

Individual proteins were spiked into various matrices at three different levels within the linear 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:

Protein	Matrix	Standard spike concentration (pg/ml)	Observed in given matrix (pg/ml)	% Recovery
CXCL8/IL-8	Pooled Donor Sera (n = 5)	1250	966.4	77%
		312.5	217.3	68%
		40	28.7	72%
CXCL8/IL-8	Pooled Donor Plasmas (n = 5)	1250	1007.8	81%
		312.5	242.8	76%
		40	28.7	72%
CXCL8/IL-8	Cell culture supernatant	1250	1291.7	103%
		312.5	349.0	109%
		40	33.5	84%
CCL5/RANTES	Pooled Donor Sera (n = 5)	ND*	---	---
CCL5/RANTES	Pooled Donor Plasmas (n = 5)	ND*	---	---
CCL5/RANTES	Cell culture supernatant	1250	1273.3	102%
		312.5	356.1	111%
		40	29.8	75%
CXCL9/MIG	Pooled Donor Sera (n = 5)	1250	779.4	62%
		312.5	186.4	58%
		40	35.8	90%
CXCL9/MIG	Pooled Donor Plasmas (n = 5)	1250	773.6	62%
		312.5	212.5	66%
		40	15.7	39%
CXCL9/MIG	Cell culture supernatant	1250	1447.8	116%
		312.5	363.6	113%
		40	36.4	91%
CCL2/MCP-1 [†]	Pooled Donor Sera (n = 5)	1250	597.0	48%
		312.5	114.5	36%
		40	5.9	15%
CCL2/MCP-1 [†]	Pooled Donor Plasmas (n = 5)	1250	543.3	43%
		312.5	110.4	34%
		40	8.9	22%
CCL2/MCP-1	Cell culture supernatant	1250	1345.7	108%
		312.5	353.2	110%
		40	31.9	80%
CXCL10/IP-10 [†]	Pooled Donor Sera (n = 5)	1250	521.6	42%
		312.5	101.5	32%
		40	26.4	66%
CXCL10/IP-10	Pooled Donor Plasmas (n = 5)	1250	969.5	78%
		312.5	279.7	87%
		40	24.4	61%
CXCL10/IP-10	Cell culture supernatant	1250	1170.2	94%
		312.5	341.5	106%
		40	38.8	97%

*ND = Not Determined. Due to the high levels of CCL5/RANTES in normal serum and EDTA-plasma samples, recovery information could not be generated for this protein.

† Recovery for these proteins is much lower than for other proteins in this assay. Variation is likely due to assay conditions and serum or plasma proteins and may affect quantitation of these proteins in serum or plasma samples.

Linearity

In two experiments, the following matrices were spiked with CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10 and were then serially diluted with Assay Diluent.

Matrix	Dilution	Observed CXCL8/IL-8 (pg/ml)	Observed CCL5/RANTES (pg/ml)	Observed CXCL9/MIG (pg/ml)	Observed CCL2/MCP-1 (pg/ml) [†]	Observed CXCL10/IP-10 (pg/ml) [‡]
Cell Culture Media	Neat	2383	2360	2558	2329	2374
	1:2	1264	1256	1397	1308	1275
	1:4	600	585	550	583	558
	1:8	306	315	327	297	317
	1:16	146	137	159	128	158
	1:32	71	62	74	68	70
	1:64	34	27	33	30	36
	1:128	20	16	18	15	19
	1:256	9	7	9	7	10
	Slope	1.00	1.07	1.03	1.06	1.00
Pooled Human Sera (n = 5)	Neat	2115		1853	1479	1324
	1:2	1212		1290	968	1087
	1:4	689		731	557	778
	1:8	292		355	260	422
	1:16	132	ND*	182	126	242
	1:32	63		85	65	129
	1:64	34		46	31	71
	1:128	17		18	14	34
	1:256	9		11	7	17
	Slope	1.01		0.97	0.99	0.81
Pooled Human Plasma (n = 5)	Neat	2226		2355	1295	3772
	1:2	1290		1393	860	2007
	1:4	717		794	510	1095
	1:8	293		365	234	584
	1:16	129	ND*	168	100	264
	1:32	63		82	44	124
	1:64	33		43	18	68
	1:128	18		21	9	34
	1:256	10		11	5	17
	Slope	1.02		0.99	1.06	0.98

*ND = Not Determined. Due to high levels of CCL5/RANTES in normal serum and EDTA-plasma samples, linearity information could not be generated for this protein.

† When testing serum samples for CXCL10/IP-10 or CCL2/MCP-1, it is recommended that the serum sample be diluted 1:10 or 1:4 respectively. This is to reduce the effects of serum interference with the quantitation of these proteins in serum samples.

Serum and Plasma Experimental Results

Serum and EDTA-plasma samples were tested using the BD CBA Human Chemokine Kit. The average values and the determined range for each protein are shown below:

Sample	Observed CXCL8/IL-8 Range (Average) pg/ml	Observed CCL5/RANTES Range (Average) pg/ml	Observed CXCL9/MIG Range (Average) pg/ml	Observed CCL2/MCP-1 Range (Average) pg/ml	Observed CXCL10/IP-10 Range (Average) pg/ml
Serum (n=10)	< 10 (< 10)	10,349–46,704 (21,839)	37–463 (145)	18–152 (77)	232–1,019 (459)
EDTA-Plasma (n=10)	< 10 (< 10)	4,382–18,783 (11,388)	48–482 (153)	< 10–57 (34)	202–1,480 (497)

Specificity

The antibody pairs used in the BD CBA Human Chemokine Kit assay have been screened for specific reactivity with their corresponding 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.

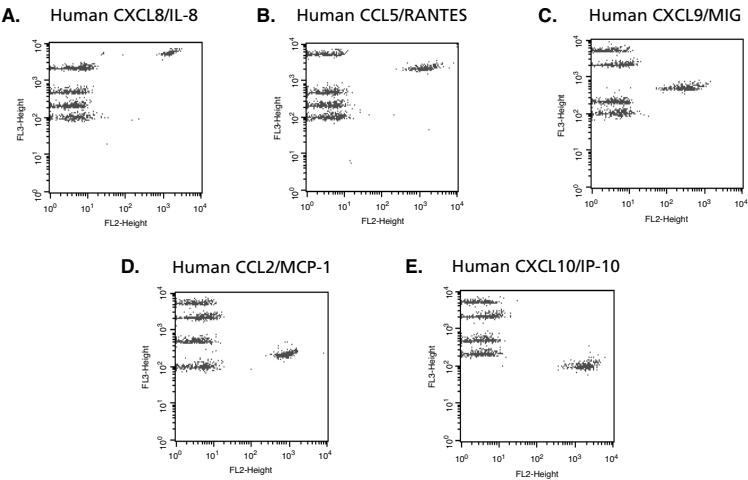


Figure 7. BD CellQuest Data for Detection of Individual Proteins

Precision

Intra-assay: Ten replicates of each of three different levels of CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10 (40, 312.5, and 1250 pg/ml) were tested.

Protein	CXCL8/IL-8			CCL5/RANTES		
Actual Mean Conc. (pg/ml):	38.7	299.0	1301.8	35.3	302.9	1319.8
SD	2.0	16.0	45.3	3.2	36.0	78.7
% CV	5.1%	5.3%	3.5%	9.1%	11.9%	6.0%

Protein	CXCL9/MIG			CCL2/MCP-1		
Actual Mean Conc. (pg/ml):	35.9	300.9	1299.4	37.4	291.3	1274.0
SD	3.1	33.5	150.1	3.6	19.7	42.5
% CV	8.7%	11.1%	11.5%	9.8%	6.8%	3.3%

Protein	CXCL10/IP-10		
Actual Mean Conc. (pg/ml):	40.0	326.4	1303.1
SD	4.4	37.3	130.8
% CV	11.0%	11.4%	10.0%

Inter-assay: Three different levels of CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, and CXCL10/IP-10 (40, 312.5, and 1250 pg/ml) were tested in four experiments conducted by different operators.

Cytokine	CXCL8/IL-8			CCL5/RANTES		
Number of Replicates:	10	10	10	10	10	10
Actual Mean Conc. (pg/ml):	36.0	297.9	1234.6	32.4	308.8	1238.1
SD	1.2	24.0	82.1	1.4	41.7	163.6
% CV	3.4%	8.1%	6.7%	4.2%	13.5%	13.2%

Cytokine	CXCL9/MIG			CCL2/MCP-1		
Number of Replicates:	10	10	10	10	10	10
Actual Mean Conc. (pg/ml):	39.2	317.5	1255.3	34.7	297.5	1275.2
SD	2.0	50.5	184.3	3.2	20.6	98.9
% CV	5.2%	15.9%	14.7%	9.2%	6.9%	7.8%

Cytokine	CXCL10/IP-10		
Number of Replicates:	10	10	10
Actual Mean Conc. (pg/ml):	39.3	305.7	1228.1
SD	1.5	42.6	160.5
% CV	3.8%	13.9%	13.1%

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

Correlation with NIBSC/WHO Standards

The NIBSC/WHO First International Standard for CXCL8/IL-8 (89/520), CCL5/RANTES (92/520) NIBSC Reference Reagent, and CCL2/MCP-1 (92/794) NIBSC Reference Reagent were evaluated using the BD CBA Human Chemokine Kit. The dose-response curves of these First International Standards paralleled the BD CBA Human Chemokine Kit standard curves for each protein. To convert sample pg/ml values determined using the BD CBA Human Chemokine Kit to the equivalent NIBSC/WHO Standard approximate pg/ml concentrations, use the conversion factors listed below:

NIBSC/WHO CXCL8/IL-8 (89/520) equivalent value (pg/ml) = $1.799 \times$ BD CBA CXCL8/IL-8 value (pg/ml)

NIBSC/WHO CCL5/RANTES (92/520) equivalent value (pg/ml) = $1.471 \times$ BD CBA CCL5/RANTES value (pg/ml)

NIBSC/WHO CCL2/MCP-1 (92/794) equivalent value (pg/ml) = $1.357 \times$ BD CBA CCL2/MCP-1 value (pg/ml)

NIBSC/WHO Reference Reagents were not available for CXCL9/MIG and CXCL10/IP-10.

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 Human Chemokine I 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 five 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.	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. Centrifuge or filter samples to reduce debris before analyzing sample with the BD CBA Human Chemokine Kit I.
Overlap of bead population fluorescence (FL3) during acquisition.	This may occur in samples with very high cytokine 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 Standards 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 BD CBA Human Chemokine Kit assay has been shown to detect Non-human Primate CXCL8/IL-8, CCL5/RANTES, and CCL2/MCP-1 proteins produced by the activation of cells from Rhesus and Cynomolgus macaques. Direct quantitation of proteins from Non-human Primates has not been validated using this Kit and results may vary.

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