

BD Cytometric Bead Array (CBA) Human Enhanced Sensitivity Master Buffer Kit Instruction Manual

Catalog No. 561521 (100 Tests)
Catalog No. 561523 (500 Tests)



Copyrights

© 2011, Becton, Dickinson and Company. All rights reserved. No part of this publication may be reproduced, transmitted, transcribed, stored in retrieval systems, or translated into any language or computer language, in any form or by any means: electronic, mechanical, magnetic, optical, chemical, manual, or otherwise, without prior written permission from BD Biosciences.

The information in this guide is subject to change without notice. BD Biosciences reserves the right to change its products and services at any time to incorporate the latest technological developments. Although this guide has been prepared with every precaution to ensure accuracy, BD Biosciences assumes no liability for any errors or omissions, nor for any damages resulting from the application or use of this information. BD Biosciences welcomes customer input on corrections and suggestions for improvement.

Trademarks

Cy is a trademark of Amersham Biosciences Corp.

FCAP Array is a trademark of Soft Flow Hungary Ltd.

Mac is a trademark of Apple Computer, Inc., registered in the US and other countries.

BD, BD Logo and all other trademarks are property of Becton, Dickinson and Company.
© 2011 BD

Regulatory information

BD flow cytometers are class I (1) laser products.

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

History

Revision	Date	Change made
23-11748-00 Rev. 01	9/2010	New document
23-11748-01 Rev. 01	1/2011	Updated Detection Reagent Diluent volumes in kit and procedure.

Contents

Chapter 1: About this kit	5
Purpose of the kit	6
Limitations	8
Kit contents	9
Storage and handling	10
Chapter 2: Before you begin	11
Workflow overview	12
Required materials	14
Chapter 3: Assay preparation	17
Preparing Human Flex Set Standards	18
Mixing Human Enhanced Sensitivity Flex Set Capture Beads	20
Diluting test samples	22
Preparing Detection Reagents (Parts A and B)	24
Chapter 4: Assay procedure	29
Performing the Human Enhanced Sensitivity Flex Set Assay	30
Data analysis	35
Chapter 5: Reference	37
Troubleshooting	38
Capture Bead and Detection Reagent Diluent	41
References	42

About this kit

This section covers the following topics:

- [Purpose of the kit \(page 6\)](#)
- [Limitations \(page 8\)](#)
- [Kit contents \(page 9\)](#)
- [Storage and handling \(page 10\)](#)

Purpose of the kit

Use of the kit The BD CBA Human Enhanced Sensitivity Flex Set System employs particles with discrete fluorescence intensities to detect soluble analytes at very low concentrations. The working assay range for most analytes in this system is 274 to 200,000 fg/mL.

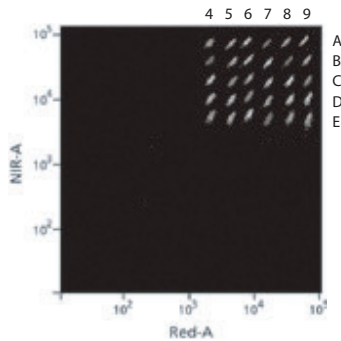
The BD CBA Human Enhanced Sensitivity Master Buffer Kit (Catalog Nos. 561521 and 561523) contains all of the supporting reagents necessary to perform an assay using a BD CBA Human Enhanced Sensitivity Flex Set. The buffers, Enhanced Sensitivity Detection Reagent (Part B), and instrument setup reagents provided in this kit have been optimized for analysis of analytes in tissue culture supernatants, plasma, and serum samples. The BD CBA Human Enhanced Sensitivity Master Buffer Kit provides sufficient reagents for the quantitative analysis of 100 samples and 10 instrument setup procedures (Catalog No. 561521) or 500 samples and 10 instrument setup procedures (Catalog No. 561523).

Principle of CBA assays BD CBA assays provide a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry.

Each capture bead in the BD CBA Human Enhanced Sensitivity Flex Set system has a distinct fluorescence and is coated with a capture antibody specific for a soluble protein. The detection reagent used in the BD CBA Human Enhanced Sensitivity Flex Set System consists of two parts. The analyte specific detection reagent (Part A) is provided with the BD CBA Human Enhanced Sensitivity Flex Set. The Enhanced Sensitivity Detection Reagent (Part B) is provided in the Master Buffer Kit. When used together, they provide a fluorescent signal in proportion to the amount of bound analyte.

When the capture beads and detection reagents are incubated with standards or unknown samples containing recognized analytes, sandwich complexes (capture bead + analyte + detection reagent) are formed. These complexes can be measured using flow cytometry to identify particles with fluorescence characteristics of both the bead and the detector.

A BD CBA Human Enhanced Sensitivity Flex Set Capture Bead is a single bead population with distinct fluorescence intensity and is coated with a capture antibody specific for a soluble protein. The bead population is resolved in two fluorescence channels of a flow cytometer. For specific instruments and the channels used to resolve the beads, see the flow cytometers listed in [Materials required but not provided \(page 14\)](#).



Each bead population is given an alphanumeric position designation indicating its position relative to other beads in the BD CBA Human Enhanced Sensitivity Flex Set System. Beads with different positions can be combined in assays to create a multiplex assay. The intensity of PE fluorescence of each sandwich complex reveals the concentration of that particular analyte. After acquiring samples on a flow cytometer, use FCAP Array™ software to generate results in graphical and tabular format.

Advantages over ELISA

The broad dynamic range of fluorescence detection via flow cytometry and the efficient capturing of multiple analytes via suspended particles enable the BD CBA Flex Set system to obtain the concentration of an unknown in substantially less time and using fewer sample dilutions compared to conventional ELISA methodology.

- The BD CBA Human Enhanced Sensitivity Flex Set assays allow for multiplexed analysis of multiple proteins from a single sample.
 - A single set of diluted standards is used to generate a standard curve for each analyte.
 - The BD CBA Human Enhanced Sensitivity Flex Set assays are more sensitive than most comparable ELISAs.
-

Limitations

Assay limitations The BD CBA Human Enhanced Sensitivity Flex Set System is not recommended for use on stream-in-air instruments for which signal intensities may be reduced, adversely affecting assay sensitivity. Stream-in-air instruments include the BD FACStar™ Plus, BD FACSVantage™, and BD Influx™ flow cytometers (BD Biosciences).

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

When several BD CBA Human Enhanced Sensitivity Flex Set assays are multiplexed, it is possible that the background (MFI of the 0 fg/mL standard point) might increase and the overall assay signals of other standard points might be reduced. This can result in lower dynamic range or loss in sensitivity in some assays. This effect might be greater as more assays are added to the multiplex.

The Human Enhanced Sensitivity Master Buffer Kit has been optimized for use with the BD CBA Human Enhanced Sensitivity Flex Sets and should not be used with Flex Sets from any other BD CBA System (soluble protein, cell signalling, etc). For an assay compatibility chart for the BD CBA Human Enhanced Sensitivity Flex Sets, please visit bdbiosciences.com/cbasetup.

Kit contents

Contents The kit contains the following components sufficient for 100 tests (Catalog No. 561521) and 500 tests (Catalog No. 561523).

Reagent	Quantity (561521)	Quantity (561523)
Assay Diluent	1 bottle, 30 mL	1 bottle, 150 mL
Capture Bead Diluent	1 bottle, 5 mL	1 bottle, 30 mL
Detection Reagent Diluent	1 bottle, 20 mL	2 bottles, 35 mL each
Wash Buffer	1 bottle, 250 mL	2 bottles, 650 mL each
Enhanced Sensitivity Detection Reagent (Part B)	2 vials, lyophilized, 50 tests each	10 vials, lyophilized, 50 tests each
Instrument Setup Bead A1	1 vial, 0.25 mL	1 vial, 0.25 mL
Instrument Setup Bead A9	1 vial, 0.25 mL	1 vial, 0.25 mL

Reagent	Quantity (561521)	Quantity (561523)
Instrument Setup Bead F1	1 vial, 1.0 mL	1 vial, 1.0 mL
Instrument Setup Bead F9	1 vial, 0.25 mL	1 vial, 0.25 mL
PE Instrument Setup Bead F1	1 vial, 0.25 mL	1 vial, 0.25 mL
PE Positive Control Detector	1 vial, 0.5 mL	1 vial, 0.5 mL

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

Storage and handling

Storage Store all kit components at 2 to 8°C. Do not freeze.

Warning All components of this kit, except for the Enhanced Sensitivity Detection Reagent (Part B), contain sodium azide. Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

2

Before you begin

This section covers the following topics:

- [Workflow overview \(page 12\)](#)
- [Required materials \(page 14\)](#)

Workflow overview

Workflow The overall workflow consists of the following steps.

Step	Description
1	Preparing Human Flex Set Standards (page 18)
2	Mixing Human Enhanced Sensitivity Flex Set Capture Beads (page 20)
3	Diluting test samples (page 22)
4	<p>Preparing Detection Reagents (Parts A and B) (page 24)</p> <p>Note: Can be prepared during the first incubation in step 6 below.</p>
5	<p>Performing instrument setup with Instrument Setup Beads, if necessary (instructions can be found at bdbiosciences.com/cbasetup)</p> <p>Note: Can be performed during one of the incubations in step 6.</p>
6	Performing the Human Enhanced Sensitivity Flex Set Assay (page 30)
7	Acquiring samples (instructions can be found at bdbiosciences.com/cbasetup)
8	Data Analysis (page 35)

Incubation times To help you plan your work, the incubation times are listed in the following table.

Procedure	Incubation time
Preparing standards	15 minutes
Preparing Enhanced Sensitivity Detection Reagent (Part B)	15 minutes
Assay Procedure	
<ul style="list-style-type: none"> ● First incubation–Capture Beads 	2 hours
<ul style="list-style-type: none"> ● Second incubation–Detection Reagent (Part A) 	2 hours
<ul style="list-style-type: none"> ● Third incubation–Enhanced Sensitivity Detection Reagent (Part B) 	1 hour

Required materials

Materials required but not provided

In addition to the reagents provided in the BD CBA Human Enhanced Sensitivity Master Buffer Kit and the BD CBA Human Enhanced Sensitivity Flex Sets, the following items are also required.

- A dual-laser flow cytometer equipped with a 488-nm or 532-nm and a 633-nm or 635-nm laser capable of distinguishing 576-nm, 660-nm, and >680-nm fluorescence. The following table lists examples of compatible instrument platforms.

Flow cytometer	Reporter channel	Bead channels
BD FACSAArray™	Yellow	Red and NIR
BD FACSCanto™ platform BD™ LSR platform BD FACSAria™ platform	PE	APC and APC-Cy™7
BD FACSCalibur™	FL2	FL4 and FL3
Note: Visit bdbiosciences.com/cbasetup for setup protocols.		

- BD Falcon™ 12 × 75-mm sample acquisition tubes for a flow cytometer (Catalog No. 352008)
- 15-mL conical polypropylene tubes (BD Falcon, Catalog No. 352097), or equivalent
- FCAP Array software (Catalog No. 641488 [PC] or 645447 [Mac®])
- Microcentrifuge

**Materials
required for
plate loader-
equipped flow
cytometers**

- Millipore MultiScreen_{HTS}-BV 1.2- μ m Clear non-sterile filter plates [Catalog No. MSBVN1210 (10 pack) or MSBVN1250 (50 pack)]
 - Millipore MultiScreen_{HTS} Vacuum Manifold (Catalog No. MSVMHTS00)
 - MTS 2/4 Digital Stirrer, IKA Works, VWR (Catalog No. 82006-096)
 - Vacuum source
 - Vacuum gauge and regulator (if not using the recommended manifold)
-

3

Assay preparation

This section covers the following topics:

- [Preparing Human Flex Set Standards \(page 18\)](#)
- [Mixing Human Enhanced Sensitivity Flex Set Capture Beads \(page 20\)](#)
- [Diluting test samples \(page 22\)](#)
- [Preparing Detection Reagents \(Parts A and B\) \(page 24\)](#)

Preparing Human Flex Set Standards

Purpose of this procedure

The BD CBA Human Enhanced Sensitivity Flex Set Standards are lyophilized and must be reconstituted and serially diluted immediately before mixing with the Capture Beads and the Detection Reagent.

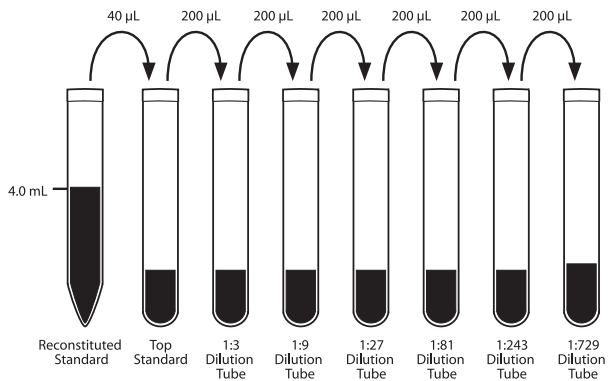
Note: You must prepare fresh standards to run with each single bead or multiplex experiment. Do not store or reuse reconstituted or diluted standards.

Procedure

To reconstitute and serially dilute the standards:

1. Open one vial of lyophilized standard from each BD CBA Human Enhanced Sensitivity Flex Set that will be tested.
2. Pool all lyophilized standard spheres into one 15-mL polypropylene tube.
3. Reconstitute the standards with 4 mL of Assay Diluent.
 - a. Allow the reconstituted standard to equilibrate for at least 15 minutes at room temperature.
 - b. Gently mix the reconstituted standard by pipet only. Do not vortex or mix vigorously.
4. Label seven 12 × 75-mm tubes and arrange them in the following order: Top Standard, 1:3, 1:9, 1:27, 1:81, 1:243, and 1:729.
5. Pipette 460 µL of Assay Diluent into the Top Standard tube.
6. Pipette 400 µL of Assay Diluent into each of the remaining 12 × 75-mm tubes.
7. Pipette 40 µL of the reconstituted standards into the Top Standard tube. Mix by gently vortexing for no longer than 3 seconds.

8. Perform a serial dilution.
 - a. Transfer 200 μL from the Top Standard to the 1:3 dilution tube and mix thoroughly by pipet only. Do not vortex.
 - b. Continue making serial dilutions by transferring 200 μL from the 1:3 tube to the 1:9 tube and so on to the 1:729 tube.



9. Prepare one 12 x 75-mm tube containing Assay Diluent to serve as the 0 fg/mL negative control.

Note: We recommend that the first eight wells or tubes in the experiment be the standards. Standards should be run in order from least concentrated (0 fg/mL) to most concentrated (Top Standard) to facilitate analysis in FCAP Array software.

Concentration of standards The approximate concentration (fg/mL) of each BD CBA Human Enhanced Sensitivity Flex Set Standard in each dilution tube is shown in the following table.

Note: See the technical data sheet for each individual assay to verify the concentration of the Top Standard.

	Dilution tube						
	Top Standard	1:3	1:9	1:27	1:81	1:243	1:729
Protein (fg/mL)	200,000	66,667	22,222	7,407	2,469	823	274

Next step Proceed to [Mixing Human Enhanced Sensitivity Flex Set Capture Beads](#) (page 20).

Mixing Human Enhanced Sensitivity Flex Set Capture Beads

Purpose of this procedure The Capture Beads provided in each BD CBA Human Enhanced Sensitivity Flex Set are at a 20X concentration and must be diluted to their optimal concentration before use.

Procedure To mix the Capture Beads:

1. Determine the number of BD CBA Human Enhanced Sensitivity Flex Sets to be used in the experiment (size of the multiplex).

- Determine the number of tests in the experiment.

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. Add an additional 2 to 3 assay tubes to the number determined.

- Vortex each Capture Bead stock vial for at least 15 seconds to resuspend the beads thoroughly.
- Determine the total volume of diluted beads needed for the experiment. Each tube/well requires 20 μL of the diluted beads. The total volume of diluted beads can be calculated by multiplying the number of tests (determined in [step 2](#)) by 20 μL .

Example: 35 tests \times 20 μL = 700 μL total bead volume

- Determine the volume needed for each capture bead. Beads are supplied so that 1.0 μL = 1 test. Therefore, the required volume (μL) of beads is equal to the number of tests.

Example: 35 tests requires 35 μL of each Capture Bead included in the assay

- Determine the volume of Capture Bead Diluent needed to dilute the beads. Calculate the Diluent volume by subtracting the volume for each bead tested from the total volume of diluted beads needed to perform the assay. See [Capture Bead and Detection Reagent Diluent \(page 41\)](#).

Example: 700 μL total volume of beads – 35 μL for each bead = volume of Capture Bead Diluent

- if testing one analyte: $700 \mu\text{L} - (35 \mu\text{L} \times 1) = 665 \mu\text{L}$ diluent
- if testing five analytes: $700 \mu\text{L} - (35 \mu\text{L} \times 5) = 525 \mu\text{L}$ diluent

7. Pipette the Capture Beads and Capture Bead Diluent into a tube labeled “Mixed Capture Beads.”
-

Next step

The Capture Beads are now ready to be transferred to the assay tubes. Discard excess prepared Capture Beads. Do not store after mixing.

If you need to dilute samples having high-protein concentrations (for example, serum or plasma samples), proceed to [Diluting test samples \(page 22\)](#). Otherwise, proceed to [Preparing Detection Reagents \(Parts A and B\) \(page 24\)](#).

If sample dilution is not required, you can save time by proceeding directly to [Performing the Human Enhanced Sensitivity Flex Set Assay \(page 30\)](#). Please note that you will need to prepare the Detection Reagents (Parts A and B) during the first assay incubation step. You will also need to perform the cytometer setup procedure during one of the incubation steps.

Diluting test samples

Purpose of this procedure

The standard curve for each BD CBA Human Enhanced Sensitivity Flex Set covers a defined set of concentrations (274 to 200,000 fg/mL). It might be necessary to dilute test samples to ensure that their median fluorescence values fall within the range of the generated standard curve. For best results, dilute samples that are known or assumed to contain high levels of a given protein. This procedure might not be required for all samples.

Procedure

To dilute samples with known high-protein concentrations:

1. Dilute the sample by the desired dilution factor (for example, 1:10 or 1:100) using the appropriate volume of Assay Diluent.

Serum or plasma samples must be diluted at least 1:3 before transferring the samples to the assay tubes or wells.

2. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing Capture Beads.
3. To facilitate analysis in FCAP Array software, load serial diluted samples in sequential wells from most concentrated to least concentrated (eg, Sample 1 – 1:4, 1:8, 1:16; Sample 2 – 1:4, 1:8, 1:16; etc).

Next step

Proceed to [Preparing Detection Reagents \(Parts A and B\) \(page 24\)](#).

Or, you can save time by proceeding directly to [Performing the Human Enhanced Sensitivity Flex Set Assay \(page 30\)](#). Please note that you will need to prepare the Detection Reagents (Parts A and B) during the first assay incubation step. If cytometer setup is required, you will also need to perform this procedure during an assay incubation step.

Preparing Detection Reagents (Parts A and B)

Purpose of the procedure

The BD CBA Human Enhanced Sensitivity Flex Set System uses a two-step detection system. The Human Detection Reagent (Part A) provided with each BD CBA Human Enhanced Sensitivity Flex Set is a 20X bulk concentration (1 μL per test). It should be mixed with other BD CBA Human Detection Reagents (Part A) and diluted to the optimal volume per test (20 μL per test) before adding the Human Detection Reagents (Part A) to a given tube or assay well.

The Enhanced Sensitivity Detection Reagent (Part B) provided in the Human Enhanced Sensitivity Master Buffer Kit is lyophilized and must be rehydrated and diluted to its optimal concentration before it can be used.

Note: Protect both Detection Reagents (Parts A and B) from exposure to direct light because they can become photobleached and will lose fluorescence intensity.

Preparing Human Detection Reagent (Part A)

To prepare Human Detection Reagent (Part A):

Note: You can use the same calculations for the number of tests and volume that you used for the Capture Beads in [Mixing Human Enhanced Sensitivity Flex Set Capture Beads \(page 20\)](#).

1. Determine the number of BD CBA Human Enhanced Sensitivity Flex Sets to be used in the experiment (size of the multiplex).
2. Determine the number of tests to be run in the experiment. Prepare a few additional tests than necessary to ensure that there is enough material prepared for the experiment.

3. Determine the total volume of diluted Human Detection Reagent (Part A) needed for the experiment. Each tube/well requires 20 μL of the diluted Human Detection Reagent. The total volume can be calculated by multiplying the number of tests (determined in [step 2](#)) by 20.

Example: $35 \text{ tests} \times 20 \mu\text{L} = 700 \mu\text{L}$ total volume

4. Determine the volume needed for each Human Detection Reagent. The Human Detection Reagent (Part A) is supplied so that $1.0 \mu\text{L} = 1$ test. Therefore, the required volume (μL) of Detection Reagent is equal to the number of tests.

Example: 35 tests requires 35 μL of each Detection Reagent included in the assay

5. Determine the volume of Detection Reagent Diluent needed to dilute the Human Detection Reagents (Part A). The volume of Detection Reagent Diluent can be calculated by subtracting the volume for each Human Detection Reagent (Part A) tested from the total volume needed. See [Capture Bead and Detection Reagent Diluent \(page 41\)](#).

Example: $700 \mu\text{L}$ total volume Detection Reagent – $35 \mu\text{L}$ for each Human Detection Reagent (Part A) = volume of Detection Reagent Diluent

- if testing one analyte: $700 \mu\text{L} - (35 \mu\text{L} \times 1) = 665 \mu\text{L}$ diluent
 - if testing five analytes: $700 \mu\text{L} - (35 \mu\text{L} \times 5) = 525 \mu\text{L}$ diluent
6. Pipette the Human Detection Reagents (Part A) and Detection Reagent Diluent into a tube labeled “Mixed Human Detection Reagents.” Store at 4°C , protected from light until ready to use.

Preparing Enhanced Sensitivity Detection Reagent (Part B)**To prepare Enhanced Sensitivity Detection Reagent (Part B):**

1. Open one vial of the lyophilized Enhanced Sensitivity Detection Reagent (Part B).
Note: This procedure outlines the steps for preparing 50 tests using one vial of Enhanced Sensitivity Detection Reagent.
 2. Reconstitute the lyophilized Enhanced Sensitivity Detection Reagent (Part B) with 0.55 mL (550 μ L) of Detection Reagent Diluent.
 - a. Incubate for 15 minutes at room temperature, protected from light.
 - b. Gently mix by pipetting up and down.
 3. Add 4.5 mL of Detection Reagent Diluent to a 15-mL conical polypropylene tube.
 4. Transfer 0.5 mL of the reconstituted Enhanced Sensitivity Detection Reagent (Part B) to the conical tube. Gently vortex.
-

Next step

Perform cytometer setup, if necessary, using the instrument setup beads. For details on setup, go to bdbiosciences.com/cbasetup and select the appropriate flow cytometer under CBA Flex Sets: Instrument Setup.

Or, if you wish to begin staining your samples for the assay, proceed to [Performing the Human Enhanced Sensitivity Flex Set Assay \(page 30\)](#), and you can perform the cytometer setup procedure during one of the incubation steps.

Note: It is not necessary to set up most digital cytometers before every experiment. Templates can be created by performing the setup and saving a template with the appropriate settings for subsequent experiments. When using a template, be sure to confirm the settings by running either setup beads or an extra well/tube from the assay to ensure that the template settings are acceptable.

4

Assay procedure

This section covers the following topics:

- [Performing the Human Enhanced Sensitivity Flex Set Assay \(page 30\)](#)
- [Data Analysis \(page 35\)](#)

Performing the Human Enhanced Sensitivity Flex Set Assay

- Before you begin**
1. Prepare the standards as described in [Preparing Human Flex Set Standards \(page 18\)](#).
 2. Mix the Capture Beads as described in [Mixing Human Enhanced Sensitivity Flex Set Capture Beads \(page 20\)](#).
 3. If necessary, dilute the unknown samples. See [Diluting test samples \(page 22\)](#).
 4. Prepare the Detection Reagents as described in [Preparing Detection Reagents \(Parts A and B\) \(page 24\)](#). You can also prepare these reagents during the first assay incubation.

Overview

Following the preparation and dilution of the individual assay components, transfer the standards or samples, mixed Capture Beads, mixed Human Detection Reagents (Part A), and Enhanced Sensitivity Detection Reagent (Part B) to the appropriate assay wells or tubes for incubation and analysis.

Note: Protect Capture Beads and both Detection Reagents (Part A and Part B) from direct exposure to light.

Assay procedure for plates

To prepare the standards and samples for analysis using plates:

1. Wet the filter plate by adding 100 μL of Wash Buffer to each well. To remove the excess volume, apply to vacuum manifold. Do not exceed 10" Hg of vacuum pressure. Aspirate for 2 to 10 seconds until wells are drained.
2. Add 50 μL of Human Flex Set Standard dilutions to the first eight wells as listed in the following table.

Well label	Concentration (fg/mL)	Standard dilution
1	0 (negative control)	no standard dilution (Assay Diluent only)
2	274	1:729
3	823	1:243
4	2,469	1:81
5	7,407	1:27
6	22,222	1:9
7	66,667	1:3
8	200,000	Top Standard

3. Add 50 μL of each unknown sample to the appropriate wells.
4. Vortex the mixed Capture Beads for at least 5 seconds.
5. Add 20 μL of the mixed Capture Beads to each assay well. Mix the plate for 5 minutes at 500 rpm using a digital shaker (do not exceed 600 rpm).
6. Incubate the plate for 2 hours at room temperature.
7. Add 20 μL of the mixed Human Detection Reagent (Part A) to each assay well. Mix the plate for 5 minutes at 500 rpm using a digital shaker.

8. Incubate the plate for 2 hours at room temperature.
9. Wash the plate twice as follows:
 - a. Apply the plate to the vacuum manifold and vacuum aspirate (do not exceed 10" Hg of vacuum pressure) for 2 to 10 seconds until the wells are drained.
 - b. Add 200 μ L of wash buffer to each assay well.
 - c. Repeat [step a](#) to vacuum aspirate the plate.
 - d. Add 200 μ L of wash buffer to each assay well.
 - e. Repeat [step a](#) to vacuum aspirate the plate.
10. Add 100 μ L of the Enhanced Sensitivity Detection Reagent (Part B) to each assay well. Mix the plate for 5 minutes at 500 rpm using a digital shaker.
11. Incubate the plate for 1 hour at room temperature.
12. Wash the plate once as follows:
 - a. Apply the plate to the vacuum manifold and vacuum aspirate (do not exceed 10" Hg of vacuum pressure) for 2 to 10 seconds until the wells are drained.
 - b. Add 200 μ L of wash buffer to each assay well.
 - c. Repeat [step a](#) to vacuum aspirate the plate.
13. Add 150 μ L of Wash Buffer to each well. Mix the plate on a digital shaker for 5 minutes at 500 rpm to resuspend beads and cells.
14. Proceed to sample acquisition. See [Next step \(page 34\)](#) for helpful information on acquisition.

**Assay procedure
for tubes**

To prepare the standards and samples for analysis using tubes:

1. Add 50 μL of Human Flex Set Standard dilutions to the first eight tubes as listed in the following table.

Tube label	Concentration (fg/mL)	Standard dilution
1	0 (negative control)	no standard dilution (Assay Diluent only)
2	274	1:729
3	823	1:243
4	2,469	1:81
5	7,407	1:27
6	22,222	1:9
7	66,667	1:3
8	200,000	Top Standard

2. Add 50 μL of each unknown sample to the appropriate assay tubes.
3. Vortex the mixed Capture Beads for at least 5 seconds.
4. Add 20 μL of the Mixed Capture Beads to each assay tube. Gently mix the tubes.
5. Incubate the tubes for 2 hours at room temperature.
6. Add 20 μL of the mixed Human Detection Reagent (Part A) to each assay tube. Gently mix the tubes.
7. Incubate the tubes for 2 hours at room temperature.
8. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200g for 5 minutes.
9. Carefully aspirate and discard the supernatant from each assay tube.

10. Add 100 μL of Enhanced Sensitivity Detection Reagent (Part B) to each assay tube. Gently mix the tubes.
 11. Incubate the tubes for 1 hour at room temperature.
 12. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200g for 5 minutes.
 13. Carefully aspirate and discard the supernatant from each assay tube.
 14. Add 300 μL of Wash Buffer to each assay tube. Vortex assay tubes briefly to resuspend beads.
-

Next step

Acquire the samples on the flow cytometer. For details, go to bdbiosciences.com/cbasetup and select the appropriate flow cytometer under CBA Flex Sets: Instrument Setup.

Acquire samples on the same day they are prepared. Prolonged storage of samples, once the assay is complete, can lead to increased background and reduced sensitivity.

To facilitate the analysis of samples in the FCAP Array software, we recommend the following guidelines:

- Acquire standards from lowest (0 fg/mL) to highest (Top Standard) concentration, followed by the test samples.
 - If running sample dilutions, acquire sequentially starting with the most concentrated sample.
 - Store all FCS files (standards and samples) in a single folder.
-

Data analysis

How to analyze data

Analyze data using FCAP Array software. For instructions on analysis, go to bdbiosciences.com/cbasetup and see the *Guide to Analyzing Data from BD CBA Kits Using FCAP Array Software*.

When analyzing the BD CBA Enhanced Sensitivity assay data with FCAP Array software, the following information might be helpful.

- The capture bead position for a BD CBA Enhanced Sensitivity assay might be different than the bead position of the same analyte in the Soluble Protein Flex Set System. Be sure to update the bead list in FCAP Array software and select the Enhanced Sensitivity Capture Bead when selecting the beads to be analyzed. An updated version of the bead list (.xml file) can be found at bdbiosciences.com/cbasetup under Tools.
- The BD CBA Enhanced Sensitivity assays measure analytes in fg/mL. When assigning standard values in FCAP Array software, a unit value must be assigned as well. If fg/mL is not available in the menu used to select the units, it can be added by clicking the **Modify List of Units** button in the lower-right area of the Table of Standards window (where the standard values are assigned).

Note: If values in pg/mL are desired, the conversion must be done after results are generated. The software will not accept standard values that are below 1.0.

- When choosing a curve fitting option, try both 4 Parameter and 5 Parameter Logistic, and select the one that results in the best curve fit (highest R^2 value).
-

Reference

This section covers the following topics:

- [Troubleshooting \(page 38\)](#)
- [Capture Bead and Detection Reagent Diluent \(page 41\)](#)
- [References \(page 42\)](#)

Troubleshooting

Recommended actions These are the actions we recommend taking if you encounter the following problems.

For best performance, vortex samples immediately before acquiring on a flow cytometer.

Problem	Recommended action
Poor standard curves	If there is no change in signal above background across the entire standard curve range, ensure that all of the components (Capture Beads, Detection Reagent, and standard) were added to each tube.
	If the curve is relatively flat and then increases at higher concentrations but not to the expected levels, make sure standards are not being vortexed or vigorously mixed while being reconstituted. The best approach is to allow the standards to equilibrate for 15 minutes in Assay Diluent prior to mixing. Mix by gently pipetting several times.
	Check that all components have been properly prepared and stored. Use freshly reconstituted standards. Ensure that incubation times are of proper length and that the assay did not sit for a prolonged period of time after the wash step.
Low event count	The beads can precipitate. Thoroughly vortex individual Capture Bead bulk vials prior to preparation of the master bead mix and vortex the master bead mix prior to dispensing into the individual assay wells. Thoroughly shake plates or vortex sample tubes prior to acquisition.
	Ensure that the stopping rule, singlet gate, and thresholds are set correctly.
	Ensure that the vacuum is not too strong and that filter membranes are not compromised (filter plates). Avoid aspiration of beads during the wash step (tubes).
Variation between duplicate samples	Vortex Capture Beads before pipetting. Beads can aggregate.

Problem	Recommended action
Little or no detection of protein in samples	Samples might be too dilute. Try various sample dilutions.
All samples are positive or above the high standard median fluorescence value	Samples might be too concentrated. Try various sample dilutions.
High background	Remove excess detection reagent by increasing the number of wash steps, since the background might be due to non-specific binding.
	Background may be produced by precipitated buffers. Check for visible precipitate and filter through a 0.2- μ m filter if necessary.
Sample dilution	We recommend diluting serum and plasma samples at least 1:3 because spike recoveries are generally better, suggesting that factors might be present at lower dilutions that inhibit the binding kinetics of the assay. If using the filter plate protocol, diluting the samples also prevents clogging of the filter membrane, which can lead to insufficient washing and high background.
Sample storage	Cytokines in general are quite labile and will degrade over time even when stored frozen at -70°C . Samples can usually be stored in single-use aliquots for 3–6 months. Sample storage strategies should be determined empirically prior to making them standard practice.

Problem	Recommended action
Biohazardous samples	It is possible to treat samples with 1% paraformaldehyde before analyzing on the flow cytometer. This might affect assay performance and should be validated. The antibody pairs are optimized for detection of native protein, so fixation should be attempted only after the samples have been incubated with the capture and detection reagents.
Anticoagulant for plasma samples	Only EDTA plasma samples have been verified by BD Biosciences.
Clogged filter plate	Serum and plasma proteins can settle and clog the membrane during incubation. Dilute samples further or perform assay incubations in a standard polystyrene U-bottom plate (Catalog No. 353910) and transfer to the filter plate immediately prior to aspiration. Resuspend the beads well prior to transfer.

Capture Bead and Detection Reagent Diluent

How to calculate Calculate the Diluent volume by subtracting the volume for each bead tested from the total volume of diluted beads needed to perform the assay. The following table lists the appropriate volumes.

No. of Flex Sets to be used	Volume per test			
	Each Capture Bead or Detection Reagent	Total Capture Bead or Detection Reagent	Capture Bead or Detection Reagent Diluent	Mixed Capture Beads or Detection Reagent
1	1 µL	1 µL	19 µL	20 µL
2	1 µL	2 µL	18 µL	20 µL
3	1 µL	3 µL	17 µL	20 µL
4	1 µL	4 µL	16 µL	20 µL
5	1 µL	5 µL	15 µL	20 µL
6	1 µL	6 µL	14 µL	20 µL
7	1 µL	7 µL	13 µL	20 µL
8	1 µL	8 µL	12 µL	20 µL
9	1 µL	9 µL	11 µL	20 µL
10	1 µL	10 µL	10 µL	20 µL
11	1 µL	11 µL	9 µL	20 µL
12	1 µL	12 µL	8 µL	20 µL
13	1 µL	13 µL	7 µL	20 µL
14	1 µL	14 µL	6 µL	20 µL
15	1 µL	15 µL	5 µL	20 µL
16	1 µL	16 µL	4 µL	20 µL
17	1 µL	17 µL	3 µL	20 µL
18	1 µL	18 µL	2 µL	20 µL
19	1 µL	19 µL	1 µL	20 µL
20	1 µL	20 µL	0 µL	20 µL

References

Related publications

1. Bishop JE, Davis KA. A flow cytometric immunoassay for β 2-microglobulin in whole blood. *J Immunol Meth.* 1997;210:79-87.
2. Camilla C, Defoort JP, Delaage M, et al. A new flow cytometry-based multi-assay system: Application to cytokine immunoassays. *Cytometry Suppl.* 1998;8:132.
3. Carson R, Vignali D. Simultaneous quantitation of fifteen cytokines using a multiplexed flow cytometric assay. *J Immunol Meth.* 1999;227:41-52.
4. Chen R, Lowe L, Wilson JD, et al. Simultaneous quantification of six human cytokines in a single sample using microparticle-based flow cytometric technology. *Clin Chem.* 1999;45:1693-1694.
5. Kricka LJ. Simultaneous multianalyte immunoassays. In: Immunoassay. Diamandis EP, Christopoulos TK, eds. Academic Press. 1996:389-404.
6. Lund-Johansen FK, Davis KJ, Bishop J, Malefyt RW. Flow cytometric analysis of immunoprecipitates: High-throughput analysis of protein phosphorylation and protein-protein interactions. *Cytometry.* 2000;39:250-259.
7. McHugh TM. Flow microsphere immunoassay for the quantitative and simultaneous detection of multiple soluble analytes. *Methods Cell Biol.* 1994;42:575-595.
8. Oliver KG, Kettman JR, Fulton RJ. Multiplexed analysis of human cytokines by use of the FlowMetrix system. *Clin Chem.* 1998;44:2057-2060.

9. Cook EB, Stahl JL, Lowe L, et al. Simultaneous measurement of six cytokines in a single sample of human tears using microparticle-based flow cytometry: allergics vs. non-allergics. *J Immunol Meth.* 2001;254:109-118.
 10. Dotti G, Salvoldo B, Takahashi S, et al. Adenovector-induced expression of human-CD40-ligand (hCD40L) by multiple myeloma cells: A model for immunotherapy. *Exp Hematol.* 2001;29:952-961.
-

United States

877.232.8995

Canada

800.268.5430

Europe

32.2.400.98.95

Japan

0120.8555.90

Asia/Pacific

65.6861.0633

Latin America/Caribbean

55.11.5185.9995



**Becton, Dickinson and Company
BD Biosciences**

San Jose, CA 95131

Toll free: 877.232.8995 (US)

Tel: 408.432.9475

Fax: 408.954.2347

bdbiosciences.com