BD Cytometric Bead Array (CBA) Mouse Enhanced Sensitivity Master Buffer Kit

Instruction Manual

Catalog No. 562246 (100 Tests)
Catalog No. 562248 (500 Tests)

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Trademarks

Regulatory information

BD flow cytometers are class 1 laser products.

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

History

Revision	Date	Change made
23-13336-00 Rev. 01	6/2011	New document

Contents

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About this kit

This section covers the following topics:

Purpose of the kit

Use of the kit

The BD™ CBA Mouse 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 Mouse Enhanced Sensitivity Master Buffer Kit (Catalog Nos. 562246 and 562248) contains all of the supporting reagents necessary to perform an assay using a BD CBA Mouse 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 and serum samples. The BD CBA Mouse Enhanced Sensitivity Master Buffer Kit provides sufficient reagents for the quantitative analysis of 100 samples and 10 instrument setup procedures (Catalog No. 562246) or 500 samples and 10 instrument setup procedures (Catalog No. 562248).

Principle of CBA assays

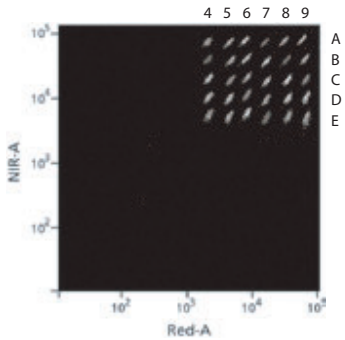
BD CBA assays provide a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry.

Each capture bead in the BD CBA Mouse 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 Mouse Enhanced Sensitivity Flex Set System consists of two parts. The analyte-specific detection reagent (Part A) is provided with the BD CBA Mouse 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 Mouse 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 Mouse 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 Mouse 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 Mouse Enhanced Sensitivity Flex Set assays are more sensitive than most comparable ELISAs.

Limitations

Assay limitations

The BD CBA Mouse 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 Mouse 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 Mouse Enhanced Sensitivity Master Buffer Kit has been optimized for use with the BD CBA Mouse 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 Mouse Enhanced Sensitivity Flex Sets, visit bdbiosciences.com/cbasetup.

Kit contents

Contents

The kit contains the following components sufficient for 100 tests (Catalog No. 562246) and 500 tests (Catalog No. 562248).

Reagent	Quantity (562246)	Quantity (562248)
Assay Diluent	1 bottle, 30 mL	1 bottle, 150 mL
Capture Bead Diluent	1 bottle, 5 mL	1 bottle, 30 mL
Detection Reagent Diluent A	1 bottle, 3 mL	1 bottle, 15 mL
Detection Reagent Diluent B	1 bottle, 20 mL	2 bottles, 35 mL each
Wash Buffer	1 bottle, 250 mL	2 bottles, 650 mL each

Reagent	Quantity (562246)	Quantity (562248)
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
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

Storage and handling

Storage

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

Warning

All components of this kit contain serum protein. 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.

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

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Before you begin

This section covers the following topics:

Workflow overview

Workflow

The overall workflow consists of the following steps.

Step	Description
1	Preparing Mouse Flex Set Standards (page 18)
2	Mixing Mouse Enhanced Sensitivity Flex Set Capture Beads (page 20)
3	Diluting test samples (page 22)
4	Preparing Detection Reagents (Parts A and B) (page 24) **Note:** Can be prepared during the first incubation in step 6 below.
5	Performing instrument setup with Instrument Setup Beads, if necessary (instructions can be found at bdbiosciences.com/cbasetup) **Note:** Can be performed during one of the incubations in step 6.
6	Performing the Mouse 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	
• First incubation–Capture Beads	2 hours
• Second incubation–Detection Reagent (Part A)	2 hours
• 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 Mouse Enhanced Sensitivity Master Buffer Kit and the BD CBA Mouse 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 FACSArray™	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
BD FACSVerse™	PE	CBA Red and CBA NIR
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. 652099 [PC] or 645447 [Mac®])
- Microcentrifuge

Materials required for plate loader-equipped flow cytometers

- Millipore MultiScreen$_{HTS}$-BV 1.2-µm 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)

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Assay preparation

This section covers the following topics:

Preparing Mouse Flex Set Standards

Purpose of this procedure

The BD CBA Mouse Enhanced Sensitivity Master Buffer Kit 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

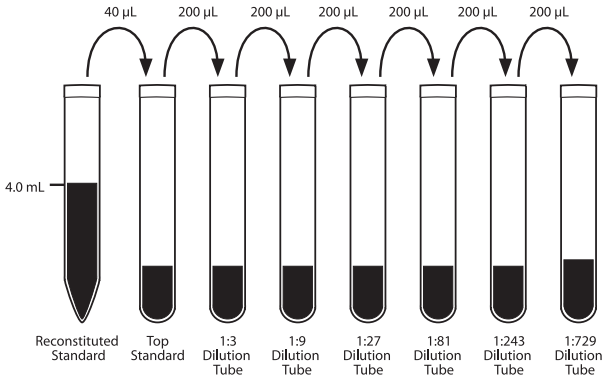
To reconstitute and serially dilute the standards:

1. Open one vial of lyophilized standard from each BD CBA Mouse 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 x 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 Mouse 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 Mouse Enhanced Sensitivity Flex Set Capture Beads (page 20).

Mixing Mouse Enhanced Sensitivity Flex Set Capture Beads

Purpose of this procedure

The Capture Beads provided in each BD CBA Mouse 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 Mouse Enhanced Sensitivity Flex Sets to be used in the experiment (size of the multiplex).
2. 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.

3. Vortex each Capture Bead stock vial for at least 15 seconds to resuspend the beads thoroughly.

4. 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 × 20 µL = 700 µL total bead volume

5. 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

6. 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 40).

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

   - if testing one analyte: 700 µL – (35 µL × 1) = 665 µL diluent
   - if testing five analytes: 700 µL – (35 µL × 5) = 525 µL 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 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 Mouse 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 Mouse 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 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 Mouse 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 Mouse Enhanced Sensitivity Flex Set System uses a two-step detection system. The Mouse Detection Reagent (Part A) provided with each BD CBA Mouse Enhanced Sensitivity Flex Set is a 20X bulk concentration (1 µL per test). It should be mixed with other BD CBA Mouse Detection Reagents (Part A) and diluted to the optimal volume per test (20 µL per test) in Detection Reagent Diluent A before adding the Mouse Detection Reagents (Part A) to a given tube or assay well.

The Enhanced Sensitivity Detection Reagent (Part B) provided in the Mouse Enhanced Sensitivity Master Buffer Kit is lyophilized and must be rehydrated and diluted to its optimal concentration in Detection Reagent Diluent B 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 Mouse Detection Reagent (Part A)

To prepare Mouse 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 Mouse Enhanced Sensitivity Flex Set Capture Beads (page 20).

1. Determine the number of BD CBA Mouse 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 Mouse Detection Reagent (Part A) needed for the experiment. Each tube/well requires 20 µL of the diluted Mouse Detection Reagent. The total volume can be calculated by multiplying the number of tests (determined in step 2) by 20.

   Example: 35 tests × 20 µL = 700 µL total volume

4. Determine the volume needed for each Mouse Detection Reagent (Part A). The Mouse Detection Reagent (Part A) is supplied so that 1.0 µL = 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 A needed to dilute the Mouse Detection Reagents (Part A). The volume of Detection Reagent Diluent A can be calculated by subtracting the volume for each Mouse Detection Reagent (Part A) tested from the total volume needed. See Capture Bead and Detection Reagent Diluent (page 40).

   Example: 700 µL total volume Detection Reagent – 35 µL for each Mouse Detection Reagent (Part A) = volume of Detection Reagent Diluent A

   - if testing one analyte: 700 µL – (35 µL × 1) = 665 µL diluent
   - if testing five analytes: 700 µL – (35 µL × 5) = 525 µL diluent

6. Pipette the Mouse Detection Reagents (Part A) and Detection Reagent Diluent A into a tube labeled "Mixed Mouse 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 µL) of Detection Reagent Diluent B.

   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 B 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. Store at 4°C, protected from light until ready to use.

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 Mouse 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.

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Assay procedure

This section covers the following topics:

Performing the Mouse Enhanced Sensitivity Flex Set Assay

Before you begin

1. Prepare the standards as described in Preparing Mouse Flex Set Standards (page 18).
2. Mix the Capture Beads as described in Mixing Mouse 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 Mouse 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 Mouse 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.

   Note: If you have not yet performed cytometer setup, you may wish to do so during this incubation.

7. Add 20 µL of the mixed Mouse 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 µL of wash buffer to each assay well.
    c. Repeat step a to vacuum aspirate the plate.
    d. Add 200 µL of wash buffer to each assay well.
    e. Repeat step a to vacuum aspirate the plate.
10. Add 100 µL 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 µL of wash buffer to each assay well.
    c. Repeat step a to vacuum aspirate the plate.
13. Add 150 µL of Wash Buffer to each well. Mix the plate on a digital shaker for 5 minutes at 500 rpm to resuspend beads.
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 Mouse 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.

   Note: If you have not yet performed cytometer setup, you may wish to do so during this incubation.

6. Add 20 µL of the mixed Mouse 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 200*g* 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 200*g* 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 *FCAP Array Software User's Guide*.

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 (ES) 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.
- Perform the FCAP Array analysis using fg/mL as the units. If values in pg/mL are desired, we recommend performing the conversion after the analysis using fg/mL in FCAP Array software. Results obtained using this method are more accurate than performing the analysis in pg/mL.
- 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).

5

Reference

This section covers the following topics:

Troubleshooting

Recommended actions

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

Note: 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. Ensure that polypropylene tubes are used.
	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 aggregate. 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-µm filter, if necessary.
Sample dilution	We recommend diluting serum 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.
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.
Clogged filter plate	Serum 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). Resuspend the beads well and transfer to the filter plate immediately prior to aspiration.

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

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