BD Cytometric Bead Array (CBA) Cell Signaling Master Buffer Kit

Instruction Manual

Catalog No. 560005 (100 Tests)
Catalog No. 560006 (500 Tests)

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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
23-12723-00 Rev. 01	2/2011	New document

Contents

1

About this kit

This section covers the following topics:

Purpose of the kit

Use of the kit

The BD™ CBA Cell Signaling Flex Sets and BD™ CBA Cell Signaling Master Buffer Kit employ particles with discrete fluorescence intensities to detect soluble analytes at very low concentrations. The working assay range for most analytes in this system is 3.9 to 1,000 units/mL.

The BD CBA Cell Signaling Master Buffer Kit contains all of the supporting reagents necessary to perform an assay using a BD CBA Cell Signaling Flex Set. The buffers and instrument setup reagents provided in this kit have been optimized to measure total or phosphorylated proteins from denatured cell lysate samples. See the technical data sheet for a given BD CBA Flex Set for actual specificity. The BD CBA Cell Signaling Master Buffer Kit provides sufficient reagents for the quantitative analysis of 100 samples and 10 instrument setup procedures (Catalog No. 560005) or 500 samples and 10 instrument setup procedures (Catalog No. 560006).

Principle of CBA assays

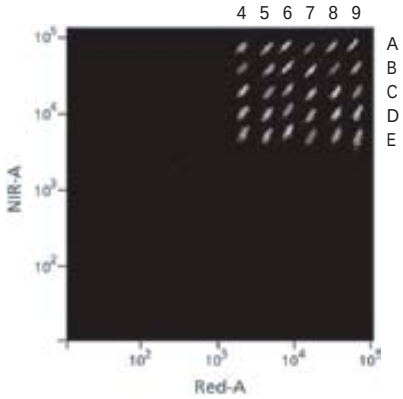
BD CBA assays provide a method of capturing a soluble analyte or set of analytes with beads of known size and fluorescence, making it possible to detect analytes using flow cytometry.

Each capture bead in the BD CBA Cell Signaling 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 Cell Signaling Flex Set System provides 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 Cell Signaling Flex Set Capture Bead is a single bead population with distinct fluorescence intensity and is coated with a capture antibody specific for an intracellular 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 15).



Each bead population is given an alphanumeric position designation indicating its position relative to other beads in the BD CBA 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.

The standard included with each Flex Set acts as a positive control for the assay and allows you to quantitate your samples in relative units per mL. Keep in mind that in most cell model systems there is considerable variation in the activation of cells from day to day. Experiments should be run with an unactivated cell sample (negative cell control) and a control activated cell sample (positive cell control). The best results are obtained when all of the relevant experimental conditions, as well as the control cells, are generated on the same day. If samples are to be run at a later date, lysates should be frozen in single-use aliquots. It is always preferable to test all samples in the same assay, since even frozen samples may experience loss of phosphorylation. Due to the biological variation in cell activation, comparing the results from experiments performed on different days can be difficult. It is, however, possible to normalize data using inter-assay positive controls.

Advantages over ELISA

The BD CBA Cell Signaling Flex Set System provides several advantages when compared to conventional ELISA and Western blot methodologies.

- The BD CBA Cell Signaling Flex Set assays allow for multiplexed analysis of multiple proteins from a single sample.
- The BD CBA Cell Signaling Flex Set assay experiment takes significantly less time than a Western blot assay and provides quantitative results.
- A single set of diluted standards is used to generate a standard curve for each analyte.

- The BD CBA Cell Signaling Flex Set assays have a wider dynamic range and are more sensitive than conventional ELISAs.

Limitations

Assay limitations

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

When several BD CBA Cell Signaling assays are multiplexed, it is possible that the background (MFI of the 0 units/mL standard point) may increase and the overall assay signals of other standard points may 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.

For assays that will be acquired on a BD FACSCalibur™ flow cytometer, we recommend that additional dilutions of the standard be prepared (ie, 1:512 and 1:1024), since it is possible that in multiplex experiments containing a large number of assays the Top Standard, 1:2, and 1:4 standard dilutions cannot be analyzed by FCAP Array software. In those cases, the Top Standard, 1:2, and 1:4 standard dilutions can be run in the experiment but might need to be excluded from the final analysis in FCAP Array software.

The BD CBA Cell Signaling Master Buffer Kit should not be used with any BD CBA Flex Sets except for the Cell Signaling Flex Set.

Kit contents

Contents

The kit contains the following components sufficient for 100 tests (Catalog No. 560005) and 500 tests (Catalog No. 560006).

Reagent	Quantity (560005)	Quantity (560006)
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, 5 mL	1 bottle, 30 mL
5X Denaturation Buffer	1 bottle, 30 mL	1 bottle, 150 mL
Wash Buffer	1 bottle, 130 mL	1 bottle, 650 mL
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

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 the 5X Denaturation Buffer, 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

Workflow

The overall workflow consists of the following steps.

Step	Description
1	Preparing test samples (page 18)
2	Preparing BD CBA Cell Signaling Flex Set Standards (page 22)
3	Mixing BD CBA Cell Signaling Flex Set Capture Beads (page 24)
4	Preparing BD CBA Cell Signaling Flex Set PE Detection Reagents (page 26) **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 BD CBA Cell Signaling 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
Assay Procedure	
• First incubation–Capture Beads	3 hours
• Second incubation–PE Detection Reagent	1 hour

Required materials

Materials required but not provided

In addition to the reagents provided in the BD CBA Cell Signaling Master Buffer Kit and the BD CBA Cell Signaling Flex Set, 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
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
- Microcentrifuge tubes (polypropylene)

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)

3

Assay preparation

This section covers the following topics:

Preparing test samples

Purpose of this procedure

BD CBA Cell Signaling Flex Sets are designed to measure total or phosphorylated proteins from denatured cell lysate samples. See the technical data sheet for a given BD CBA Flex Set for actual specificity. You must lyse and denature cell samples using the 5X Denaturation Buffer provided in the BD CBA Cell Signaling Master Buffer Kit before use in a BD CBA Cell Signaling Flex Set assay.

The standard curve for each BD CBA Cell Signaling Flex Set covers a defined set of concentrations from 3.9 to 1,000 units/mL. It might be necessary to dilute test samples to ensure that their mean 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.

In cases in which the samples are known or assumed to contain low levels of a given protein, lyse the sample in a lower volume of 5X -Denaturation Buffer, thereby concentrating the protein in the sample. It is important that the cell number or the total protein concentration of the cell lysate sample is known so that results determined using the BD CBA Cell Signaling Flex Sets can be normalized (eg, units/mL/10^6 cells or units/mL/µg of cell lysate).

BD Biosciences offers a CBA Flex Set against GAPDH (Catalog No. 560792). By running the BD CBA Human GAPDH Flex Set together with the BD CBA Flex Sets specific for phosphorylated and total signaling proteins, you will be able to normalize your phosphorylation results to the amount of lysate added.

Before you begin

Heat the 5X Denaturation Buffer to 37°C before use (shake or vortex until all precipitates are in solution).

Note: To denature the cell lysate, it is important that the final concentration of the Denaturation Buffer is 1X after being mixed with cells. The process is the same as preparing a sample for gel electrophoresis and Western blotting except that Denaturation Buffer is used instead of SDS-PAGE sample buffer.

Procedure for cells in suspension

1. Count the cells in the sample to get an approximate idea of the protein concentration, which should be greater than 1 mg/mL. The protein concentration depends on the cell type (eg, Jurkat = 100–200 µg/10^6 cells, while peripheral blood lymphocytes [PBLs] = 25–50 µg/10^6 cells).
2. Treat the cells to induce or inhibit protein phosphorylation, as required for the experiment.
3. Use one of the following methods to prepare samples for denaturation:
   - Halt activation of the cells by adding the appropriate amount of 5X Denaturation Buffer so that the final concentration of Denaturation Buffer is 1X.
   - Add ice-cold PBS to the activated cells and pellet by centrifugation. Add an appropriate amount of 1X Denaturation Buffer (prepared by diluting the 5X Denaturation Buffer with DI water) to resuspend the cell pellet.

- Add ice-cold lysis buffer containing a detergent (eg, Triton® X-100, NP40, etc) to the cells. Incubate for 15 to 30 minutes at 4°C and pellet the insoluble material by centrifugation. Transfer the supernatant to a clean tube and add the appropriate amount of 5X Denaturation Buffer so that the final concentration of Denaturation Buffer is 1X.

Note: Regardless of the method used, recoveries can be enhanced by adding protease inhibitors and phosphatase inhibitors.

4. Denature the sample by immediately placing it in a boiling water bath for 5 minutes. The sample might be very viscous and difficult to pipette due to the presence of DNA. This can be remedied by one of the following methods:

   - Shear the DNA using a probe sonicator. The sample should be sonicated until it is easy to pipette and the liquid falls as discrete drops.
   - Pass the sample through a 26-gauge needle several times.
   - Add a very high quality (protease-free) DNase I to the denatured sample. DNase I is the best solution if many different lysates will be tested. However, a high quality DNase I must be used, or residual proteases in the DNase I will destroy the samples.

5. Determine the protein concentration.
6. Cell lysates can be stored in aliquots at –70°C for up to 6 months at this point. If samples are stored frozen, thaw the samples before proceeding to step 7. Avoid multiple freeze/thaw treatments of samples. Centrifuge samples at 14,000 rpm for 3 minutes to pellet the debris before use.

7. Dilute cell lysate samples by the desired dilution factor (ie, 1:2, 1:10, or 1:20) using the appropriate volume of Assay Diluent. Samples must be diluted at least 1:4 to reduce the percentage of SDS and should not contain more than 20 µg of total protein.

Procedure for adherent cells

1. Count the cells before plating to get an approximate idea of the protein concentration, which should be greater than 1 mg/mL.
2. Treat the cells to induce or inhibit protein phosphorylation, as required for the experiment.
3. Use one of the following methods to prepare samples for denaturation:
   - Halt activation of the cells by adding the appropriate amount of 5X Denaturation Buffer so that the final concentration of Denaturation Buffer is 1X.
   - Aspirate off all liquid and add 1X Denaturation Buffer (prepared by diluting the 5X Denaturation Buffer with DI water) to lyse the cells. Scrape or agitate the cells to dislodge them from the plate.

   Note: Regardless of the method used, recoveries can be enhanced by adding protease inhibitors and phosphatase inhibitors.
4. Follow step 4 through step 7 in Procedure for cells in suspension (page 19).

Next step

Proceed to Preparing BD CBA Cell Signaling Flex Set Standards (page 22).

Preparing BD CBA Cell Signaling Flex Set Standards

Purpose of this procedure

The BD CBA Cell Signaling Flex Set Standards are lyophilized and must be reconstituted and serially diluted immediately before mixing with the Capture Beads and the PE Detection Reagent. Each Cell Signaling Flex Set Standard was assigned an arbitrary unit value. In each case, the unit potency of the BD CBA Flex Set Standard will be kept consistent from lot to lot.

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

Procedure

To reconstitute and serially dilute the standards:

1. Open the standard and transfer the lyophilized sphere to a 1.5-mL microcentrifuge tube.
2. Reconstitute the standard with 100 µL of Assay Diluent.
3. Warm the tube to 37°C and vortex.

   Note: Once reconstituted, the standard should be stored at 4°C and is stable for 3 months. If you are using a reconstituted standard, warm it to 37°C and vortex.

4. Label nine 12 × 75-mm tubes and arrange them in the following order: Top Standard, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256.
5. Add 20 µL of each BD CBA Cell Signaling Flex Set Standard to be run in the experiment to the Top Standard tube.
6. Add Assay Diluent (yellow buffer) to the Top Standard tube to bring the final volume to 1 mL.

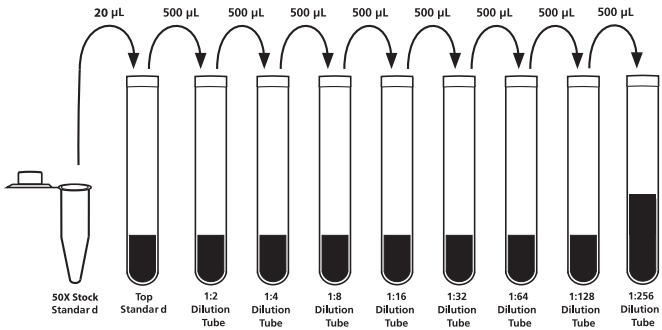
Example: If five BD CBA Cell Signaling Flex Sets are being multiplexed for a given experiment, you will add 20 µL of each BD CBA Cell Signaling Flex Set Standard to the Top Standard tube (5 × 20 µL = 100 µL standard volume). Then add 900 µL of Assay Diluent (1 mL final volume – 100 µL [volume of standards] = 900 µL Assay Diluent).

7. Add 500 µL of Assay Diluent into each of the remaining 12 x 75-mm tubes.

8. Perform a serial dilution.

   a. Transfer 500 µL from the Top Standard to the 1:2 dilution tube and mix thoroughly.

   b. Continue making serial dilutions by transferring 500 µL from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube.



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

Note: We recommend that the first 10 wells or tubes in the experiment be the standards. Standards should be run in order from least concentrated (0 U/mL) to most concentrated (Top Standard) to facilitate analysis in FCAP Array software.

Concentration of standards

The approximate concentration (units/mL) of each BD CBA Cell Signaling 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								
Cell Signaling Flex Set	**Top Standard**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**
Protein (units/mL)	1,000	500	250	125	62.5	31.25	15.6	7.8	3.9

Next step

Proceed to Mixing BD CBA Cell Signaling Flex Set Capture Beads (page 24).

Mixing BD CBA Cell Signaling Flex Set Capture Beads

Purpose of this procedure

The Capture Beads provided in each BD CBA Cell Signaling Flex Set are at a 50X concentration and must be diluted to their optimal concentration before use.

Mixing the beads

To mix the Capture Beads:

1. Determine the number of BD CBA Cell Signaling 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 50 µL of the diluted beads. Calculate the total volume of diluted beads by multiplying the number of tests (determined in step 2) by 50 µL.

   Example: 35 tests × 50 µL = 1,750 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) for more examples.

   Example: 1,750 µL total volume of beads – 35 µL for each bead = volume of Capture Bead Diluent

   - if testing one analyte: 1,750 µL – (35 µL × 1) = 1,715 µL diluent
   - if testing five analytes: 1,750 µL – (35 µL × 5) = 1,575 µL diluent

7. Pipette the Capture Beads and Capture Bead Diluent into a tube labeled "Mixed Capture Beads." Protect from light until ready to use.

Next step

The Capture Beads are now ready for use in the assay. Discard excess prepared Capture Beads. Do not store after mixing.

Proceed to Preparing BD CBA Cell Signaling Flex Set PE Detection Reagents (page 26).

Or, to save time you can proceed directly to Performing the BD CBA Cell Signaling Flex Set Assay (page 30). Please note that you will need to prepare the PE Detection Reagents during the first assay incubation step. You will also need to perform the cytometer setup procedure during one of the incubation steps.

Preparing BD CBA Cell Signaling Flex Set PE Detection Reagents

Purpose of this procedure

The PE Detection Reagent provided with each BD CBA Cell Signaling Flex Set is a 50X bulk concentration. It should be mixed with other BD CBA Cell Signaling Flex Set PE Detection Reagents and diluted to the optimal volume per test (50 µL per test) before adding the PE Detection Reagents to a given tube or assay well.

Note: Protect the PE Detection Reagents from exposure to direct light. They can become photobleached and will lose fluorescence intensity.

Preparing PE Detection Reagent

To prepare PE Detection Reagent:

Note: You can use the same calculations for the number of tests and volume that you used for the Capture Beads in Mixing BD CBA Cell Signaling Flex Set Capture Beads (page 24).

1. Determine the number of BD CBA Cell Signaling Flex Sets to be used in each assay tube or well 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 PE Detection Reagent (blue buffer) needed for the experiment. Each tube/well requires 50 µL of the diluted PE Detection Reagent. Calculate the total volume by multiplying the number of tests (determined in step 2) by 50. Allow the PE Detection Reagent to come to room temperature, and mix it well before use. The solution is somewhat viscous so be careful that you pipette the proper volume.

   Example: 35 tests × 50 µL = 1,750 µL total volume
4. Determine the volume needed for each PE Detection Reagent. The PE Detection Reagent is supplied so that 1.0 µL = 1 test. Therefore, the required volume (µL) of PE 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 PE Detection Reagents. 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) for more examples.

Example: 1,750 µL total volume Detection Reagent – 35 µL for each Detection Reagent = volume of Detection Reagent Diluent

- if testing one analyte: 1,750 µL – (35 µL × 1) = 1,715 µL diluent
- if testing five analytes: 1,750 µL – (35 µL × 5) = 1,575 µL diluent

6. Pipette the Detection Reagents and Detection Reagent Diluent into a tube labeled "Mixed PE Detection Reagents." 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 BD CBA Cell Signaling 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 BD CBA Cell Signaling Flex Set Assay

Before you begin

1. Prepare samples. See Preparing test samples (page 18).
2. Prepare the standards as described in Preparing BD CBA Cell Signaling Flex Set Standards (page 22).
3. Mix the Capture Beads as described in Mixing BD CBA Cell Signaling Flex Set Capture Beads (page 24).
4. Prepare the Detection Reagents as described in Preparing BD CBA Cell Signaling Flex Set PE Detection Reagents (page 26). 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, and mixed PE Detection Reagents to the appropriate assay wells or tubes for incubation and analysis.

Note: Protect Capture Beads and PE Detection Reagents from direct exposure to light.

Assay procedure for plates

To prepare the standards and samples for analysis using plates:

1. Add 50 µL of BD CBA Cell Signaling Flex Set Standard dilutions to the first 10 wells as listed in the following table.

Well label	Standard dilution	Concentration (units/mL)
1	no standard dilution (Assay Diluent only)	0 (negative control)
2	1:256	3.9
3	1:128	7.8
4	1:64	15.6
5	1:32	31.25
6	1:16	62.5
7	1:8	125
8	1:4	250
9	1:2	500
10	Top Standard	1,000

2. Mix the unknown sample dilutions thoroughly and add 50 µL of each unknown sample to the appropriate wells.

   To facilitate analysis in FCAP Array software, load serial diluted samples in sequential wells from most concentrated to least concentrated (eg, Sample 1 – 1:2, 1:4, 1:8; Sample 2 – 1:2, 1:4, 1:8, etc).

3. Vortex the mixed Capture Beads for at least 5 seconds and add 50 µL of the Capture Beads to each assay well. Mix the plate for 5 minutes at 500 rpm using a digital shaker (do not exceed 600 rpm).

4. Incubate the plate for 3 hours at room temperature, protected from light.

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

5. Add 50 µL of the mixed PE Detection Reagent to each well. Mix the plate for 5 minutes at 500 rpm using a digital shaker (do not exceed 600 rpm).

   Note: Once the PE Detection Reagent is added, the liquid in each well will appear green.

6. Incubate the plate for 1 hour at room temperature, protected from light.
7. 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.
8. Add 150 µL of Wash Buffer to each assay well. Mix the plate for 5 minutes at 500 rpm using a digital shaker to resuspend the beads.
9. 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 BD CBA Cell Signaling Flex Set Standard dilutions to the first 10 tubes as listed in the following table.

Well label	Standard dilution	Concentration (units/mL)
1	no standard dilution (Assay Diluent only)	0 (negative control)
2	1:256	3.9
3	1:128	7.8
4	1:64	15.6
5	1:32	31.25

Well label	Standard dilution	Concentration (units/mL)
6	1:16	62.5
7	1:8	125
8	1:4	250
9	1:2	500
10	Top Standard	1,000

2. Mix the unknown sample dilutions thoroughly and add 50 µL of each unknown sample to the appropriate assay tubes.
3. Vortex the mixed Capture Beads for at least 5 seconds and add 50 µL of the Capture Beads to each assay tube. Gently mix the tubes.
4. Incubate the tubes for 3 hours at room temperature, protected from light.

   Note: If you have not yet performed cytometer setup, you may wish to do so during this incubation.
5. Add 50 µL of the mixed PE Detection Reagent to each assay tube. Gently mix the tubes.

   Note: Once the PE Detection Reagent is added, the liquid in each well will appear green.
6. Incubate the tubes for 1 hour at room temperature, protected from light.
7. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200*g* for 5 minutes.
8. Carefully aspirate and discard the supernatant from each assay tube.
9. Add 300 µL of Wash Buffer to each assay tube. Vortex the assay tubes briefly to resuspend the 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 FCAP Array software, we recommend the following guidelines:

- Acquire standards from lowest (0 units/mL) to highest (Top Standard) concentration, followed by the test samples.
- If running sample dilutions, acquire sequentially starting with the most concentrated sample (eg, Sample 1 – 1:2, 1:4, 1:8; Sample 2 – 1:2, 1:4, 1:8, etc).
- 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 Cell Signaling Flex Set assay data with FCAP Array software and 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.
	Reconstitute lyophilized standards in polypropylene tubes.
	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. The beads can aggregate.
Little or no detection of protein in samples	Samples might be too dilute. Try various sample dilutions.
	Samples might not be denatured or activation may not have been successful.

Problem	Recommended action
All samples are positive or above the high standard mean fluorescence value	Samples might be too concentrated. Try various sample dilutions.
High background	Samples might be too concentrated. Try various sample dilutions.
	Remove excess Detection Reagent by increasing the number of wash steps, since the background might be due to non-specific binding.
	Background can be produced by precipitated buffers. Check for visible precipitate and filter through a 0.2-µm filter, if necessary.
Sample dilution	Samples should be diluted at least 1:4 because the concentration of SDS in the 1X Denaturation Buffer needs to be reduced for optimal binding.
No correlation of Western blot and BD CBA data	Sample concentration is important. The BD CBA beads will saturate when incubated with 10–20 µg of total protein. If a greater amount of total protein is required to see a band, then there may be issues correlating with BD CBA data.
Activation issues	Perform a time course to ensure activation times are appropriate.
	Add Denaturation Buffer to a final concentration of 1X and immediately transfer to a 37°C water bath to stop activation.
Clogged filter plate	Cellular debris and non-denatured DNA can settle and clog the membrane during incubation. Spin lysates to remove debris and see Preparing test samples (page 18) for instructions on how to denature DNA. 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	49 µL	50 µL
2	1 µL	2 µL	48 µL	50 µL
3	1 µL	3 µL	47 µL	50 µL
4	1 µL	4 µL	46 µL	50 µL
5	1 µL	5 µL	45 µL	50 µL
6	1 µL	6 µL	44 µL	50 µL
7	1 µL	7 µL	43 µL	50 µL
8	1 µL	8 µL	42 µL	50 µL
9	1 µL	9 µL	41 µL	50 µL
10	1 µL	10 µL	40 µL	50 µL
11	1 µL	11 µL	39 µL	50 µL
12	1 µL	12 µL	38 µL	50 µL
13	1 µL	13 µL	37 µL	50 µL
14	1 µL	14 µL	36 µL	50 µL
15	1 µL	15 µL	35 µL	50 µL
16	1 µL	16 µL	34 µL	50 µL
17	1 µL	17 µL	33 µL	50 µL
18	1 µL	18 µL	32 µL	50 µL
19	1 µL	19 µL	31 µL	50 µL
20	1 µL	20 µL	30 µL	50 µL

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
21	1 µL	21 µL	29 µL	50 µL
22	1 µL	22 µL	28 µL	50 µL
23	1 µL	23 µL	27 µL	50 µL
24	1 µL	24 µL	26 µL	50 µL
25	1 µL	25 µL	25 µL	50 µL
26	1 µL	26 µL	24 µL	50 µL
27	1 µL	27 µL	23 µL	50 µL
28	1 µL	28 µL	22 µL	50 µL
29	1 µL	29 µL	21 µL	50 µL
30	1 µL	30 µL	20 µL	50 µL
31	1 µL	31 µL	19 µL	50 µL
32	1 µL	32 µL	18 µL	50 µL
33	1 µL	33 µL	17 µL	50 µL
34	1 µL	34 µL	16 µL	50 µL
35	1 µL	35 µL	15 µL	50 µL
36	1 µL	36 µL	14 µL	50 µL
37	1 µL	37 µL	13 µL	50 µL
38	1 µL	38 µL	12 µL	50 µL
39	1 µL	39 µL	11 µL	50 µL
40	1 µL	40 µL	10 µL	50 µL
41	1 µL	41 µL	9 µL	50 µL
42	1 µL	42 µL	8 µL	50 µL
43	1 µL	43 µL	7 µL	50 µL
44	1 µL	44 µL	6 µL	50 µL
45	1 µL	45 µL	5 µL	50 µL
46	1 µL	46 µL	4 µL	50 µL
47	1 µL	47 µL	3 µL	50 µL
48	1 µL	48 µL	2 µL	50 µL
49	1 µL	49 µL	1 µL	50 µL
50	1 µL	50 µL	0 µL	50 µL

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