BD™ Cytometric Bead Array

Cell Signaling Master Buffer Kit

Instruction Manual

Cat. No. 560005 100 Tests

Cat. No. 560006 500 Tests

BD

Kit Contents

560005, 100 tests

(Store the following items at 4°C)

Assay Diluent: 1 bottle 30 mL

Capture Bead Diluent: 1 bottle 5 mL

Detection Reagent Diluent: 1 bottle 5 mL

5× Denaturation Buffer: 1 bottle 30 mL

Wash Buffer: 1 bottle 130 mL

Instrument Setup Bead A1: 1 vial 0.25 mL

Instrument Setup Bead A9: 1 vial 0.25 mL

Instrument Setup Bead F1: 1 vial 1.0 mL

Instrument Setup Bead F9: 1 vial 0.25 mL

PE Instrument Setup Bead F1: 1 vial 0.25 mL

PE* Positive Control Detector: 1 vial 0.5 mL

Kit Contents

560006, 500 tests

(Store the following items at 4°C)

Assay Diluent: 1 bottle 150 mL

Capture Bead Diluent: 1 bottle 30 mL

Detection Reagent Diluent: 1 bottle 30 mL

5× Denaturation Buffer: 1 bottle 150 mL

Wash Buffer: 1 bottle 650 mL

Instrument Setup Bead A1: 1 vial 0.25 mL

Instrument Setup Bead A9: 1 vial 0.25 mL

Instrument Setup Bead F1: 1 vial 1.0 mL

Instrument Setup Bead F9: 1 vial 0.25 mL

PE Instrument Setup Bead F1: 1 vial 0.25 mL

PE* Positive Control Detector: 1 vial 0.5 mL

Table of Contents

Introduction

Flow cytometry is an analysis tool that allows for the discrimination of different particles on the basis of size and flourescence. The BD™ Cytometric Bead Array (CBA) Cell Signaling Flex Sets and Cell Signaling Master Buffer Kits employ particles with discrete fluorescence intensities to detect soluble analytes. The BD™ CBA is combined with flow cytometry to create a powerful particle-based immunoassay.

The BD CBA Cell Signaling Flex Set system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure a soluble analyte. Each bead in a BD CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate. The BD CBA Cell Signaling Flex Set capture bead is in suspension to allow for the detection of an analyte in a small sample volume. The combined advantages of the broad dynamic range of fluorescent detection via flow cytometry and the efficient capturing of multiple analytes via suspended particles enable the BD CBA Flex Set system to use fewer sample dilutions and to obtain the value of an unknown in substantially less time (compared to conventional ELISA and western blot techniques).

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 for use on BD flow cytometers.

Note: The BD CBA Cell Signaling Master Buffer Kit should not be used with any non-Cell Signaling BD CBA Flex Sets.

Principle of the Test

A BD™ CBA Cell Signaling Flex Set capture bead is a single bead population with a distinct fluorescence intensity and is coated with a capture antibody specific for an intracellular protein. The bead population is resolvable in the NIR and Red channels of a BD FACSArray™ bioanalyzer or the FL3 and FL4 channels of a BD FACSCalibur™ flow cytometer.

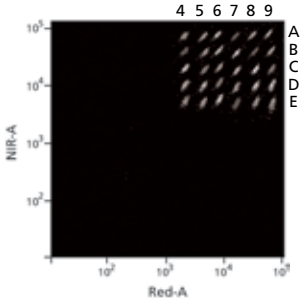


Figure 1

Each bead population is given an alpha-numeric position designation indicating its position relative to other beads in the BD CBA Flex Set system. Beads with different positions can be combined to create a multiplex assay. In a BD CBA Flex Set assay the capture bead, PE-conjugated detection reagent, and standard or test samples are incubated together to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results are generated in graphical and tabular formats using the FCAP Array™ software. The BD CBA Cell Signaling Master Buffer Kit provides sufficient reagents for the analysis of 100 samples and 10 instrument setup procedures (Cat. No. 560005) or 500 samples and 10 instrument setup procedures (Cat. No. 560006).

The standard included with each Flex Set acts as a positive control for the assay and it allows the user to quantitate their samples in relative Units/ml. It should be kept 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, as even frozen samples may experience loss of phosphorylation. Due to the biological variation in cell activation, direct comparison of results from experiments performed on different days can be difficult. It is, however, possible to normalize data using inter-assay positive controls.

Advantages

The BD CBA Cell Signaling Flex Set system provides several advantages when compared with 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.
- A BD CBA Cell Signaling Flex Set assay experiment takes significantly less time than a western blot assay and provides quantitative results.
- The BD CBA Cell Signaling Flex Set assays have a wider dynamic range than conventional ELISAs.

Limitations

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

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 may be greater as more assays are added to the multiplex.

For assays that will be acquired on a BD FACSCalibur flow cytometer, it is recommended that additional dilutions of the standard be prepared (ie, 1:512 and 1:1024) as it is possible that in multiplex experiments containing a large number of assays the Top Standard, 1:2, and 1:4 standard dilutions will not be analyzable by the FCAP Array software. In those cases, the Top Standard, 1:2, and 1:4 standard dilutions can be run in the experiment but may need to be excluded from the final analysis in the FCAP Array software. Please see the ***BD FACSCalibur setup manual*** for further limitations.

Warnings and Precautions

All components of this kit contain phosphate buffered solution containing protein* and 0.09% sodium azide. Sodium azide yields a highly toxic hydrazoic acid under acidic conditions. Avoid exposure to skin and eyes, ingestion, and contact with heat, acids, and metals. Wash exposed skin with soap and water. Flush eyes with water. Dilute azide compounds in running water before discharging to avoid accumulation of potentially explosive deposits in plumbing.

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

Materials Required but not Provided

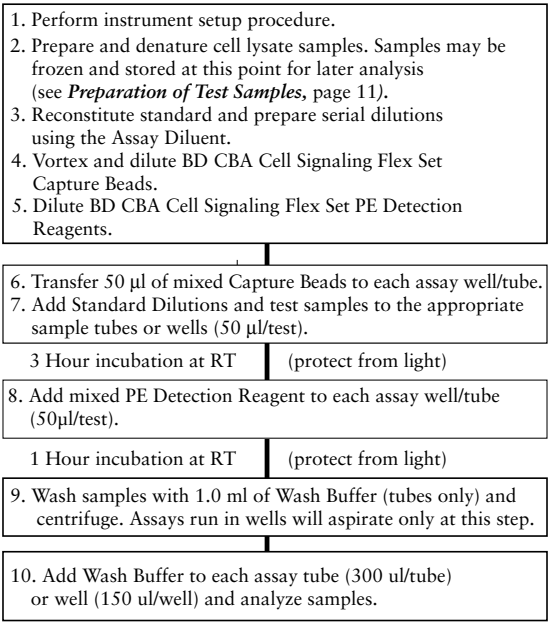
In addition to the reagents provided in the BD™ CBA Cell Signaling Master Buffer Kit and the BD CBA Cell Signaling Flex Sets, the following items are also required:

- 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. Refer to ***Appendix***, Table 2 for examples of compatible instrument platforms.
- 12 × 75 mm sample acquisition tubes for a flow cytometer (eg, BD Falcon™ Cat. No. 352008).
- FCAP Array™ Software (Cat. No. 338621).
- BD CaliBRITE™ 3 Beads, (Cat. No. 340486) to run experiments on a BD FACSCalibur.
- BD CaliBRITE™ APC Beads, (Cat. No. 340487) to run experiments on a BD FACSCalibur.
- BD FACSComp™ Software to run experiments on a BD FACSCalibur.
- Microcentrifuge.
- Microcentrifuge tube (polypropylene).

Required for Plate-loader-equipped Flow Cytometers

- Standard microtiter plate for BD FACSArray Bioanalyzer Setup (BD Falcon™ Cat. No. 353910).
- Millipore MultiScreen$_{HTS}$-BV 1.2 µm Clear non-sterile filter plates, [Cat. No. MSBVN1210 (10 pack) or MSBVN1250 (50 pack)].
- Millipore MultiScreen$_{HTS}$ Vacuum Manifold, (Cat. No. MSVMHTS00).
- MTS 2/4 digital Stirrer, IKA Works, VWR, (Cat. No. 82006-096).
- Vacuum source.
- Vacuum gauge and regulator (if not using recommended manifold).

Overview: BD CBA Cell Signaling Flex Set Assay Procedure



Perform Instrument Setup

In order to ensure that the flow cytometer is performing optimally, perform the instrument setup procedure prior to preparing the Flex Set assay. Refer to the appropriate flow cytometry instrument setup manual included in this kit for instructions on how to setup your instrument.

Preparation of Test Samples

BD™ CBA Cell Signaling Flex Sets are designed to measure total or phosphorylated proteins from denatured cell lysate samples (refer to the technical data sheet for a given BD CBA Flex Set for actual specificity). It is necessary to lyse and denature cell samples using the 5× 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 1000 Units/ml. It may be necessary to dilute test samples to ensure that their mean fluorescence values fall within the limits or range of the generated standard curve. For best results, samples that are known or assumed to contain high levels of a given protein should be diluted as described below. In cases where the samples are known or assumed to contain low levels of a given protein, the sample should be lysed in a lower volume of lysis 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). It is necessary to heat the 5× Denaturation Buffer to 37°C before use (shake or vortex until all precipitates have gone back into solution). To denature the cell lysate, it is important that the final concentration of the Denaturation Buffer is 1× after being mixed with cells.

The process is basically the same as preparing a sample for gel electrophoresis and western blotting except that Denaturation Buffer is used instead of SDS-PAGE sample buffer.

In order 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.).

Cells in Suspension

1. Count cells in sample. This gives an approximate idea of protein concentration, which should be greater than 1 mg/mL (protein concentration is dependent on cell type, eg, Jurkat = 100 – 200 µg/10^6 cells while peripheral blood lymphocytes [PBL] = 25 – 50 µg/10^6 cells).
2. Treat 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 5× Denaturation Buffer so that the final concentration of Denaturation Buffer is 1×.
   - Add ice-cold PBS to the activated cells and pellet by centrifugation. Add an appropriate amount of 1× Denaturation Buffer (prepared by diluting the 5× Denaturation Buffer with 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 – 30 minutes at 4°C and pellet insoluble material by centrifugation. Transfer the supernatant to a clean tube and add the appropriate amount of 5× Denaturation Buffer so that the final concentration of Denaturation Buffer is 1×.

     Note: Regardless of the method used, recoveries may be enhanced by adding protease inhibitors and phosphatase inhibitors.
4. Denature sample by immediately placing in a boiling water bath for 5 minutes. The sample may be very viscous and difficult to pipet 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 pipet 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 protein concentration.
6. Cell lysates may be stored in aliquots at –70°C for up to 6 months at this point. If samples are stored frozen, thaw sample before proceeding to Step 7. Avoid multiple freeze/thaw treatments of sample. Samples should be centrifuged at 14,000 rpm for 3 minutes before use to pellet debris.
7. Dilute cell lysate sample by the desired dilution factor (ie, 1:2, 1:10, or 1:20) using the appropriate volume of Assay Diluent. Sample must be diluted at least 1:4 to reduce the percentage of SDS and should not contain more than 20 µg of total protein.
8. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing Capture Beads.

Adherent Cells

1. Count cells before plating. This is to give an approximate idea of protein concentration, which should be greater than 1 mg/mL.
2. Treat 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 5× Denaturation Buffer so that the final concentration of Denaturation Buffer is 1×.
   - Aspirate off all liquid and add 1× Denaturation Buffer (prepared by diluting the 5× Denaturation Buffer with water) to lyse the cells. Scrape or agitate cells to dislodge from plate.

     Note: Regardless of the method used, recoveries may be enhanced by adding protease inhibitors and phosphatase inhibitors.
4. Denature sample by immediately placing in a boiling water bath for 5 minutes. The sample may be very viscous and difficult to pipet 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 pipet 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 protein concentration.
6. Cell lysates may be stored in aliquots at –70°C for up to 6 months at this point. If samples are stored frozen, thaw sample before proceeding to Step 7. Avoid multiple freeze/thaw treatments of sample. Samples should be centrifuged at 14,000 rpm for 3 minutes before use to pellet debris.
7. Dilute cell lysate sample by the desired dilution factor (ie, 1:2, 1:10, or 1:20) using the appropriate volume of Assay Diluent. Sample must be diluted at least 1:4 to reduce the percentage of SDS and should not contain more than 20 µg of total protein.
8. Mix sample dilutions thoroughly before transferring samples to the appropriate assay tubes containing Capture Beads.

Preparation of BD CBA Cell Signaling Flex Set Standards

The standard provided with each BD CBA Cell Signaling Flex Set is provided in a lyophilized form as a 50× bulk recombinant protein (50,000 Units/mL) and should be serially diluted before mixing with the Capture Beads and the PE Detection Reagent for a given assay. Each Cell Signaling BD CBA 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.

1. Transfer the lyophilized sphere to a 1.5 mL microfuge tube. Reconstitute the standard by adding 100 µL of assay diluent, warming the tube to 37°C, and vortexing. Once reconstituted, the standard should be stored at 4°C and is stable for 3 months. If you are using a reconstituted standard, simply warm to 37°C and vortex to mix well.
2. Label 12 × 75 mm tubes (BD Falcon™, **Cat. No. 352008**) and arrange them in the following order: Top Standard, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256.
3. Add 20 µL of each Cell Signaling BD CBA Flex Set Standard to be run in the experiment to the Top Standard tube.
4. Add Assay Diluent (yellow buffer) to the Top Standard tube to bring the final volume to 1 mL.

   Example: If 5 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 total volume) and will then add 900 µL of Assay Diluent (1 mL Assay Diluent - 100 µL [volume of standards added] = 900 µL Assay Diluent).
5. Add 500 µL of Assay Diluent to each of the remaining tubes.
6. Perform a serial dilution by transferring 500 µL from the Top Standard to the 1:2 dilution tube and mix thoroughly. 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 and mix thoroughly. The Assay Diluent serves as the negative control.
7. It is recommended that the first ten 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).

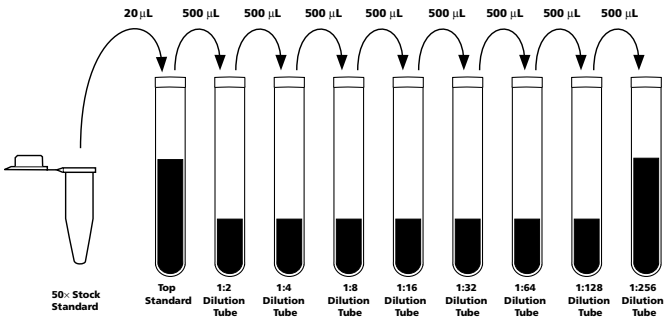


Figure 2. Preparation of BD™ CBA Cell Signaling Flex Set Standard Dilutions

The typical concentration (Units/mL) of each BD CBA Cell Signaling Flex Set Standard in each dilution tube is shown in ***Table 1***.

Table 1. BD™ CBA Cell Signaling Flex Set Standard concentrations after dilution

BD CBA Cell Signaling Flex Set Standard	Top Standard	1:2 Dilution Tube	1:4 Dilution Tube	1:8 Dilution Tube	1:16 Dilution Tube	1:32 Dilution Tube	1:64 Dilution Tube	1:128 Dilution Tube	1:256 Dilution Tube
Protein (Units/mL)	1000	500	250	125	62.5	31.25	15.6	7.8	3.9

Preparation of BD CBA Cell Signaling Flex Set Capture Beads

The Capture Beads provided in each BD CBA Cell Signaling Flex Set are at a 50× concentration and must be diluted to their optimal concentration before adding to a given assay tube or assay well.

1. Determine the number of BD CBA Cell Signaling Protein Flex Sets to be used in the experiment (size of the multiplex).
2. Determine the number of tests in the experiment. It is recommended that the user prepare a few additional tests than they will use in the experiment to ensure that there is enough material prepared for the experiment.
3. Vortex each Capture Bead stock vial for at least 15 seconds to resuspend beads thoroughly.
4. Determine the total volume of diluted beads needed for the experiment. Each tube/well requires 50 µL of the diluted beads. The total volume of diluted beads can be calculated by multiplying the number of tests (determined in step 2 above) by 50 µL.
   - eg, 35 tests × 50 µL = 1750 µL total volume of diluted beads.
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.
   - eg, 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. The volume of Capture Bead Diluent can be calculated by subtracting the volume for each bead tested from the total volume of diluted beads needed to perform the assay. Refer to ***Appendix, Table 4*** for more examples.
   - eg, 1750 µL total volume of diluted beads - 35 µL for each bead = volume of Capture Bead Diluent.
   - eg, if testing one analyte: 1750 µL – (35 µL × 1) = 1715 µL diluent.
   - eg, if testing 5 analytes: 1750 µL – (35 µL × 5) = 1575 µL diluent.
7. Pipette the Capture Beads and Capture Bead Diluent into a tube labeled Mixed Capture Beads.

Preparation of BD CBA Cell Signaling Flex Set PE Detection Reagents

The PE Detection Reagent provided in each BD CBA Cell Signaling Flex Set is at a 50× concentration and must be diluted to its optimal concentration before adding to a given assay tube or well. The calculations below should be the same as in the previous section. This section can be performed during the initial 3 hour incubation of capture beads with samples/standards.

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

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 to be run in the experiment. It is recommended that the user prepare a few additional tests than they will use in the experiment 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 your experiment. Each tube/well requires 50 µL of the diluted PE Detection Reagent. The total volume of diluted PE can be calculated by multiplying the number of tests (calculated above) by 50 µL. The PE Detection Reagent should be brought to room temperature and mixed well before use. The solution is somewhat viscous so care should be taken in making sure that the proper volume is pipetted.
   - eg, 35 tests × 50 µL = 1750 µL total volume of PE.
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.
   - eg, 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. The volume of Detection Reagent Diluent can be calculated by subtracting the volume for each PE Detection Reagent tested from the total volume of PE needed. Refer to ***Appendix, Table 4*** for more examples.
   - eg, 1750 µL total volume PE - 35 µL for each Detection Reagent = volume of Detection Reagent Diluent.
   - eg, if testing one analyte: 1750 µL – (35 µL × 1) = 1715 µL diluent.
   - eg, if testing 5 analytes: 1750 µL – (35 µL × 5) = 1575 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.

BD CBA Cell Signaling Flex Set Assay Procedure

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.

For Plates:

1. Prepare all reagents as described in previous sections before starting the experiment.
2. Vortex the Mixed Capture Beads for at least 5 seconds. Add 50 µL of the Mixed Capture Beads to each assay well.
3. Add 50 µL of Standard or sample to the assay wells.
4. Mix the microwell plate for 5 minutes using a digital shaker at 500 RPM (do not exceed 600 RPM) and incubate plate for 3 hours at RT and protect from direct exposure to light.
5. Add 50 µL of the Mixed PE Detection Reagent to each assay well. Mix the microwell plate for 5 minutes using a digital shaker at 500 RPM (do not exceed 600 RPM) and incubate the plate for 1 hour at RT and protect from direct exposure to light.

   Note: Once the Mixed PE Detection Reagent is added, the liquid in each well should appear green in color.
6. Apply the plate to the vacuum manifold and vacuum aspirate (do not exceed 10" Hg of vacuum) until wells are drained (2 – 10 seconds).
7. Add 150 µL of Wash Buffer to each assay well. Shake microwell plate on a digital shaker at 500 RPM for 5 minutes to resuspend beads.
8. Begin analyzing samples on a flow cytometer. Proceed to the appropriate flow cytometry instrument instruction manual for acquiring the BD CBA Flex Sets.

   Note: Prolonged storage of samples, once the assay is complete, can lead to increased background and reduced sensitivity.

For Tubes:

1. Prepare all reagents as described in previous sections before starting the experiment.
2. Vortex the Mixed Capture Beads for at least 5 seconds. Add 50 µL of the Mixed Capture Beads to each assay tube.
3. Add 50 µL of Standard or sample to the assay tubes.
4. Mix assay tubes gently and incubate plate for 3 hours at RT and protect from direct exposure to light.
5. Add 50 µL of the Mixed PE Detection Reagent to each assay tube. Mix the tubes gently and incubate for 1 hour at RT and protect from direct exposure to light.

   Note: Once the Mixed PE Detection Reagent is added, the liquid in each well should appear green in color.
6. Add 1.0 mL of Wash Buffer to each assay tube and centrifuge at 200 x g for 5 minutes.
7. Carefully aspirate and discard the supernatant from each assay tube.
8. Add 300 µL of Wash Buffer to each assay tube. Vortex tubes briefly to resuspend beads.
9. Begin analyzing samples on a flow cytometer. It is recommended that each tube be mixed gently before analyzing on the flow cytometer. Proceed to the appropriate flow cytometry instrument instruction manual for acquiring the BD CBA Flex Sets.

   Note: Prolonged storage of samples, once the assay is complete, can lead to increased background and reduced sensitivity.

Troubleshooting Tips

Problem	Suggested Solution
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 were 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 master bead mix and vortex the master bead mix prior to dispensing into the individual assay wells. Thoroughly shake plate or vortex sample tubes prior to acquisition.
	Ensure that the stopping rule, singlet gate, and thresholds are set correctly.
	Ensure that vacuum is not too strong and that filter membranes are not compromised (filter plates). Avoid aspiration of beads during wash step (tubes).
Variation between duplicate samples	Vortex capture beads before pipetting. Beads can aggregate.
Little or no detection of protein in samples	Samples may be too dilute. Try various sample dilutions.
	Samples may not be denatured or activation may not have been successful.
All samples are positive or above the high standard mean fluorescence value	Samples may be too concentrated Try various sample dilutions.
High background	Test various sample dilutions, the sample may be too concentrated. Remove excess PE detection reagent by increasing the number of wash steps as the background may 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	Samples shoud 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 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 in order 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 refer to page 11 or 12 for instructions on how to denature DNA. Dilute samples further or perform assay incubations in a standard polystyrene U-bottom plate (BD 353910) and transfer to the filter plate immediately prior to aspiration. Resuspend the beads well prior to transfer.

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Appendix

Table 2. Recommended Instrument Platforms

Flow Cytometer	Reporter Channel	Bead Channel
BD FACSArray™	Yellow	Red and NIR
BD FACSCanto™ II* BD™ LSR II* BD FACSAria™*	PE	APC and APC-Cy7
BD FACSCalibur™	FL2	FL4 and FL3

*Please visit **bdbiosciences.com/flexset** for setup protocol.

Table 3. Essential control assay tubes

Tube No.	Reagents (All reagent volumes are 50 µl)
1 Negative Control	Capture Beads, Assay Diluent, PE Detection Reagent
2 3.9 U/mL Standard	Capture Beads, Standard 1:256 Dilution, PE Detection Reagent
3 7.8 U/mL Standard	Capture Beads, Standard 1:128 Dilution, PE Detection Reagent
4 15.6 U/mL Standard	Capture Beads, Standard 1:64 Dilution, PE Detection Reagent
5 31.25 U/mL Standard	Capture Beads, Standard 1:32 Dilution, PE Detection Reagent
6 62.5 U/mL Standard	Capture Beads, Standard 1:16 Dilution, PE Detection Reagent
7 125 U/mL Standard	Capture Beads, Standard 1:8 Dilution, PE Detection Reagent
8 250 U/mL Standard	Capture Beads, Standard 1:4 Dilution, PE Detection Reagent
9 500 U/mL Standard	Capture Beads, Standard 1:2 Dilution, PE Detection Reagent
10 1000 U/mL Standard	Capture Beads, Standard "Top Standard", PE Detection Reagent

Table 4. Capture Bead and PE Detection Reagent Diluent Calculations

No. of Flex Sets to be used	Volume of each Capture Bead or PE Detection Reagent/test	Total Capture Bead volume/test	Volume of Capture Bead or Detection Reagent Diluent/test	Total volume of mixed Capture Beads or PE Detection Reagents/test
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
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

Notes

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