BD Cytometric Bead Array (CBA) Human Immunoglobulin Master Buffer Kit

Instruction Manual

Catalog No. 558683 (100 Tests)

BD

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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
23-12724-00 Rev. 01	4/2011	New document
23-12724-01	8/2012	Updated dilution instructions for human serum samples.

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 Human Immunoglobulin Flex Set System includes the BD™ CBA Human Immunoglobulin Master Buffer Kit and the following BD CBA Human Immunoglobulin Flex Set Assays: Total IgG, IgG_2, IgG_3, IgG_4, IgM, and IgA, all available separately.

The BD CBA Human Immunoglobulin Flex Sets and Human Immunoglobulin Master Buffer Kit employ particles with discrete fluorescence intensities to detect soluble immunoglobulin.

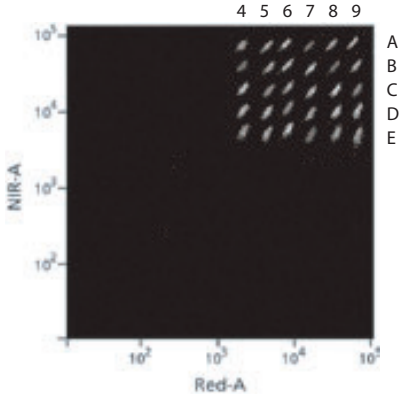
The BD CBA Human Immunoglobulin Master Buffer Kit (Catalog No. 558683) contains all of the supporting reagents necessary to perform an assay using a BD CBA Human Immunoglobulin Flex Set. The buffers and instrument setup reagents provided in this kit have been optimized for analysis of immunoglobulin in serum samples. The BD CBA Human Immunoglobulin Master Buffer Kit provides sufficient reagents for the quantitative analysis of 100 samples and 10 instrument setup procedures.

Principle of CBA assays

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

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

A BD CBA Human Immunoglobulin Flex Set Capture Bead is a single bead population with distinct fluorescence intensity and is coated with a capture antibody specific for an immunoglobulin subclass. 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).



Note: The BD CBA Human Immunoglobulin assays reside on a subset of these bead populations.

Each bead population is given an alphanumeric position designation indicating its position relative to other beads in the BD CBA Human Immunoglobulin 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 BD CBA Human Immunoglobulin Flex Set System provides several advantages when compared with conventional ELISA:

- Flex Set assays allow for multiplexed analysis of multiple proteins from a single sample.
- Flex Set assays have a wider dynamic range than conventional ELISAs.
- Flex Set assays use a human serum–based standard, resulting in a standard curve that is more linear with sample dilutions than a standard curve generated using recombinant protein.
- BD CBA Human Immunoglobulin Flex Set Standards have been calibrated against the international reference preparation CRM-470.[1,2]

Limitations

Assay limitations

The BD CBA Human Total IgG assay cannot be multiplexed with any other assay. It must be run as a single plex.

The BD CBA Human IgE assay is not compatible with the BD CBA Human Immunoglobulin assay system. IgE can be measured using the BD CBA Human Soluble Protein assay.

Multiplexing of BD CBA Human Immunoglobulin Flex Set assays with assays from the other BD CBA Flex Set Systems is not supported.

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

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

When several BD CBA Human Immunoglobulin Flex Set assays are multiplexed, it is possible that the background (MFI of the 0-ng/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 may 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 some experiments 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 may need to be excluded from the final analysis in FCAP Array software.

The buffers contained in the BD CBA Human Immunoglobulin Master Buffer Kit have been formulated specifically for use with the BD CBA Human Immunoglobulin Flex Sets. This buffer kit should not be used with any non-Human Immunoglobulin BD CBA Flex Sets. In addition, do not attempt to substitute any buffers from another non-immunoglobulin Master Buffer Kit, since this could lead to poor assay performance.

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

Kit contents

Contents

The kit contains the following components sufficient for 100 tests.

Reagent	Quantity
Assay Diluent	2 bottles, 40 mL each
Capture Bead Diluent	1 bottle, 5 mL
Detection Reagent Diluent	1 bottle, 5 mL
Wash Buffer	1 bottle, 260 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

Reagent	Quantity
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

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 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.

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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 Human Ig Flex Set Standards (page 18)
2	Mixing Human Ig Flex Set Capture Beads (page 22)
3	Diluting test samples (page 24)
4	Preparing Human Ig 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 Human Immunoglobulin 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
Assay procedure	
• First incubation–Capture Beads	1 hour
• Second incubation–Detection Reagent	2 hours

Required materials

Materials required but not provided

In addition to the reagents provided in the BD CBA Human Immunoglobulin Master Buffer Kit and the BD CBA Human Immunoglobulin 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
Note: Visit bdbiosciences.com/cbasetup for setup protocols.		

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

Materials required for plate loader-equipped flow cytometers

- Millipore MultiScreen$_{HTS}$-BV 1.2-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 Human Ig Flex Set Standards

Purpose of this procedure

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

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

All of the assays in the BD CBA Human Immunoglobulin Flex Set System use the same lyophilized standard. This means that for any assay, single plex or multiplex, only one standard vial needs to be reconstituted. The dilutions performed to obtain the Top Standard for some of the assays are different. For this reason, some of the assays cannot be run in a multiplex. See Limitations (page 8) for more information.

For each assay, single bead or multiplex, a single standard curve needs to be prepared.

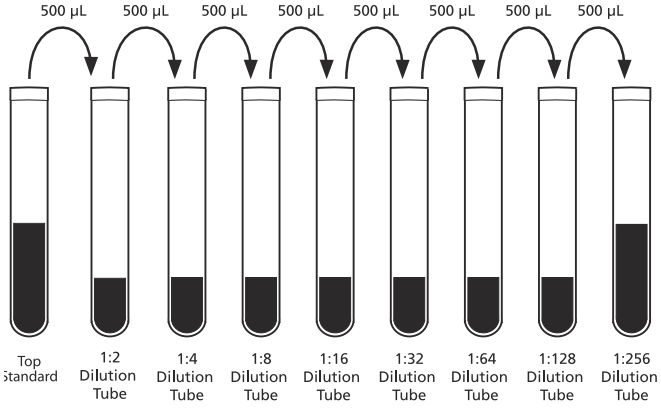
Procedure

To reconstitute and serially dilute the standards:

1. Open one vial of lyophilized standard from one of the BD CBA Human Immunoglobulin Flex Sets that will be tested.
2. Transfer the lyophilized standard sphere into a 15-mL polypropylene tube.
3. Reconstitute the standards with 1.0 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 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. Create the assay-specific Top Standard.
    a. Transfer the reconstituted standard to the Top Standard tube.
        - For Total Human IgG assays, transfer 50 µL of the reconstituted standard.
        - For Human IgG_2, IgG_3, IgG_4, IgM, and IgA assays (single or multiplex combinations), transfer the entire contents of the reconstituted standard directly to the Top Standard tube. It is already at the correct dilution.
    b. For Total Human IgG assays, add 1.55 mL of Assay Diluent to the Top Standard tube.
    c. Mix the Top Standard tube by pipet only. Do not vortex or mix vigorously.
6. Pipette 500 µL of Assay Diluent into each of the remaining 12 × 75-mm tubes.
7. Perform a serial dilution.
    a. Transfer 500 µL from the Top Standard to the 1:2 dilution tube and mix thoroughly by pipet only. Do not vortex.

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.



8. Prepare one 12 × 75-mm tube containing Assay Diluent to serve as the 0-ng/mL negative control.

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

Concentration of standards

The approximate concentration (ng/mL) of each BD CBA Human Immunoglobulin 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.

Note: IgG_2, IgG_3, IgG_4, IgM, and IgA can be multiplexed. Total IgG must be run as an individual assay (single plex).

	Concentration (ng/mL) in each dilution tube								
Assay	**Top Stand.**	**1:2**	**1:4**	**1:8**	**1:16**	**1:32**	**1:64**	**1:128**	**1:256**
IgG_2	3400.0	1700.0	850.0	425.0	212.5	106.3	53.1	26.6	13.3
IgG_3	1150.0	575.0	287.5	143.8	71.9	35.9	18.0	9.0	4.5
IgG_4	400.0	200.0	100.0	50.0	25.0	12.5	6.3	3.1	1.6
IgM	910.0	455.0	227.5	113.8	56.9	28.4	14.2	7.1	3.6
IgA	2730.0	1365.0	682.5	341.3	170.6	85.3	42.7	21.3	10.7
Total IgG	430.0	215.0	107.5	53.8	26.9	13.4	6.7	3.4	1.7

Next step

Proceed to Mixing Human Ig Flex Set Capture Beads (page 22).

Mixing Human Ig Flex Set Capture Beads

Purpose of this procedure

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

Procedure

To mix the Capture Beads:

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

   Note: Keep in mind that not all of the Human Immunoglobulin Flex Sets can be multiplexed. See Limitations (page 8) for more information.

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. The total volume of diluted beads can be calculated 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).

   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."

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, proceed to Diluting test samples (page 24). Otherwise, proceed to Preparing Human Ig Flex Set PE Detection Reagents (page 26).

Diluting test samples

Purpose of this procedure

The standard curve for each BD CBA Human Immunoglobulin Flex Set covers a defined set of concentrations. 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, serially dilute samples. When analyzing, determine which dilutions fall within the linear portion of the curve, correct for dilution factors, and average the results. Typical serum ranges for each of the human immunoglobulins have been previously reported in the literature.[1,3] See the table of suggested starting sample dilutions for each assays in Starting dilutions for human serum samples (page 25).

Procedure

To dilute samples with known high-Ig concentrations:

1. Dilute the sample by the desired dilution factor (eg, 1:10 or 1:100) using the appropriate volume of Assay Diluent. Serial dilutions can be performed from the initial dilution using Assay Diluent.
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 – 1:4,000; 1:8,000; 1:16,000; Sample 2 – 1:4,000; 1:8,000; 1:16,000, etc).

Starting dilutions for human serum samples

The following table provides suggested starting dilutions for human serum samples. With normal human serum, these dilutions will most likely fall near the top of the linear range of the assay. We recommend that these initial dilutions be followed by additional serial dilutions to ensure that at least some sample dilutions fall within the linear portion of the curve. All dilutions should be performed using the Assay Diluent provided in this kit.

Total IgG	IgG_2	IgG_3	IgG_4	IgM	IgA
1:30,000	1:2,000	1:2,000	1:2,000	1:2,000	1:2,000

A series of dilutions will be required to arrive at a final dilution of 1:30,000. We recommend the following dilution scheme to ensure that there is sufficient Assay Diluent for all samples. Additional Assay Diluent may be purchased separately (Catalog No. 560104).

Tube	Sample	Assay diluent	Final dilution
A	10 µL of sample	240 µL	1:25
B	10 µL from tube A	240 µL	1:625
C	10 µL from tube B	230 µL	1:15,000
D	120 µL from tube C	120 µL	1:30,000

Next step

Proceed to Preparing Human Ig Flex Set PE Detection Reagents (page 26).

Or, you can save time by proceeding directly to Performing the Human Immunoglobulin Flex Set Assay (page 30). Please note that you will need to prepare the PE Detection Reagents 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 Human Ig Flex Set PE Detection Reagents

Purpose of the procedure

The PE Detection Reagent provided with each BD CBA Human Immunoglobulin Flex Set is a 50X bulk concentration (1 µL per test). It should be mixed with other BD CBA Human Immunoglobulin 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.

Procedure

To prepare Human 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 Human Ig Flex Set Capture Beads (page 22).

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

   Note: Keep in mind that not all of the Human Ig Flex Sets can be multiplexed. See Limitations (page 8).

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 needed for the experiment. Each tube/well requires 50 µL of the diluted PE Detection Reagent. The total volume can be calculated by multiplying the number of tests (determined in step 2) by 50.

   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 PE Detection Reagent tested from the total volume of diluted PE needed. See Capture Bead and Detection Reagent Diluent (page 40).

   Example: 1,750 µL total volume PE 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 PE Detection Reagents and Detection Reagent Diluent into a tube labeled "Mixed Human 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 Human Immunoglobulin Flex Set Assay (page 30), and you can perform the cytometer setup procedure during one of the incubation steps.

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

4

Assay procedure

This section covers the following topics:

Performing the Human Immunoglobulin Flex Set Assay

Before you begin

1. Prepare the standards as described in Preparing Human Ig Flex Set Standards (page 18).
2. Mix the Capture Beads as described in Mixing Human Ig Flex Set Capture Beads (page 22).
3. Dilute the unknown samples. See Diluting test samples (page 24).
4. Prepare the Detection Reagents as described in Preparing Human Ig 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 the Capture Beads and the PE Detection Reagents 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 the vacuum manifold. Do not exceed 10'' Hg of vacuum pressure. Aspirate for 2 to 10 seconds until wells are drained.

2. Add 50 µL of Human Ig Flex Set Standard dilutions to the first 10 wells as listed in the following table.

Well label	Standard dilution	Concentration (ng/mL)
1	no standard dilution (Assay Diluent only)	0 (negative control)
2	1:256	The concentration of the Top Standard and each dilution varies for each Ig. See the Concentration of standards (page 21) for approximate concentrations.
3	1:128	
4	1:64	
5	1:32	
6	1:16	
7	1:8	
8	1:4	
9	1:2	
10	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 and add 50 µL of the mixed Capture Beads to each assay well.
5. Mix the plate for 5 minutes at 500 rpm using a digital shaker (do not exceed 600 rpm).
6. Incubate the plate for 1 hour at room temperature.

   Note: If you have not performed cytometer setup, you may wish to do so during this incubation or the incubation in step 9.

7. Wash the plate 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.
8. Add 50 µL of the mixed PE Detection Reagent to each assay well. Mix the plate for 5 minutes at 500 rpm using a digital shaker.
9. Incubate the plate for 2 hours at room temperature.
10. Wash the plate 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 150 µL of Wash Buffer to each assay well.
    c. Repeat step a to vacuum aspirate the plate.
11. Add 150 µL of Wash Buffer to each well. Mix the plate on a digital shaker for 5 minutes at 500 rpm to resuspend the beads.
12. Proceed to sample acquisition. See Next step (page 34) for helpful information on acquisition.

Assay procedure for tubes

To prepare the standards and samples for analysis using tubes:

1. Add 50 µL of Human Ig Flex Set Standard dilutions to the first 10 tubes as listed in the following table.

Well label	Standard dilution	Concentration (ng/mL)
1	no standard dilution (Assay Diluent only)	0 (negative control)
2	1:256	The concentration of the Top Standard and each dilution varies for each Ig. See the Concentration of standards (page 21) for approximate concentrations.
3	1:128	
4	1:64	
5	1:32	
6	1:16	
7	1:8	
8	1:4	
9	1:2	
10	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 and add 50 µL of the Capture Beads to each assay tube.
4. Gently mix the tubes and incubate for 1 hour at room temperature.

   Note: If you have not performed cytometer setup, you may wish to do so during this incubation or the incubation in step 8.

5. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200*g* for 5 minutes.

6. Carefully aspirate and discard the supernatant from each tube.
7. Add 50 µL of the mixed PE Detection Reagent to each tube.
8. Gently mix the tubes and incubate for 2 hours at room temperature.
9. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200*g* for 5 minutes.
10. Carefully aspirate and discard the supernatant from each assay tube.
11. 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 FCAP Array software, we recommend the following guidelines:

- Acquire standards from lowest (0 ng/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 – 1:4,000; 1:8,000; 1:16,000; Sample 2 – 1:4,000; 1:8,000; 1:16,000, 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 Human Ig 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.
	If the curve is relatively flat and then increases at higher concentrations but not to the expected levels, make sure standards are not being vortexed or vigorously mixed while being reconstituted. The best approach is to allow the standards to equilibrate for 15 minutes in Assay Diluent prior to mixing. Mix by gently pipetting several times.
	Check that all components have been properly prepared and stored. Use freshly reconstituted standards. Ensure that incubation times are of proper length and that the assay did not sit for a prolonged period of time after the wash step.
Low event count	The beads can 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 aspirating the 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.
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 Beads and detection reagents.
Clogged filter plate	Serum and plasma proteins can settle and clog the membrane during incubation. Dilute samples further or perform assay incubations in a standard polystyrene U-bottom plate (Catalog No. 353910) and transfer to the filter plate immediately prior to aspiration. Resuspend the beads well prior to transfer.

Capture Bead and Detection Reagent Diluent

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

No. of Flex Sets to be used	Volume per test			
	Each Capture Bead or Detection Reagent	Total Capture Bead or Detection Reagent	Capture Bead or Detection Reagent Diluent	Mixed Capture Beads or Detection Reagent
1	1 µL	1 µL	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

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