BD™ Cytometric Bead Array (CBA)

Functional Bead Conjugation Buffer Set

Instruction Manual

Cat. No. 558556 15 Reactions

BD

Table of Contents

Introduction

Flow cytometry is an analysis tool that allows for the discrimination of different particles on the basis of size and fluorescence. The BD™ CBA Functional Bead system consists of different color-coded 7.5 µm polystyrene beads that can be classified using a flow cytometer (Refer to ***Appendix***, table 1 for examples of compatible instrument platforms).

The Functional Bead Conjugation Buffer Set contains the supporting reagents necessary to covalently link water-soluble proteins to the surface of functional beads. The beads, once linked to a protein, can be used as a capture matrix for analytes of interest. The Functional Bead Conjugation Buffer Set contains enough material for 15 coupling reactions. Each coupling reaction yields approximately $3 – 6 \times 10^6$ beads which is enough material to run 500 or 1000 tests at a concentration of 6,000 beads per well. For a 500 test conjugation reaction 90 µg of protein is needed, and for a 1000 test conjugation reaction 180 µg of protein is needed. After covalently linking protein to the beads, it is recommended that a test be performed to confirm the success of the reaction. For that purpose, BD Biosciences offers three different phycoerythrin (PE) Functional Bead Ig Detectors that can be used to confirm the presence of antibody covalently linked to the beads. (See page 8 for ordering information). If another type of protein was coupled to the functional beads, please refer to the ***Conjugation Confirmation*** section (pg. 11) for options.

Principle

The Functional Bead Conjugation Buffer Set is used to covalently link water-soluble proteins to the surface of color-coded functional beads. The bond between the bead and the protein is formed using sulfo-SMCC chemistry. Before attempting to conjugate a protein to the beads, it is important to ensure the protein has been purified and is free of BSA, glycine, Tris, or any other proteins or protein stabilizing additives. Presence of stabilizing or contaminating proteins may affect the performance of the beads after conjugation. The conjugation method will activate and bind any free amino group in the sample to the beads. For a successful conjugation the protein needs to be suspended in PBS, pH 7.2 ± 0.2.

The procedure to conjugate a protein to a functional bead consists of 4 major steps:

- Step I – Bead Preparation
- Step II – Protein Modification
- Step III – Buffer Exchange to Remove Unreacted Components
- Step IV – Protein Conjugation

The estimated time to completion for the entire conjugation procedure is approximately 3.5 hrs.

Once the beads are conjugated with a protein, it is recommended that the success of the reaction be confirmed. If the functional bead was conjugated with an antibody, it is recommended that the BD Biosciences Functional Bead Ig Detectors be used to confirm the presence of antibody bound to the beads (See ***Supporting Reagents*** section for catalog numbers). The procedure to confirm the conjugation of an antibody will take approximately 45 minutes.

If the protein cannot be identified using one of the Functional Bead Ig Detectors, then a biotinylated antibody specific to that protein can be used in conjunction with streptavidin-PE (see ***Supporting Reagents*** section for catalog number). A flow cytometer (refer to ***Appendix***, table 1 for examples of compatible instrument platforms) will be used to analyze any sample.

Advantages

- Simple conjugation procedure completed in less than 4 hours.
- Conjugation reaction requires less then 100 µg of protein at a concentration of 1 mg/mL.
- Multiple bead populations that can be used individually or multiplexed as needed (See ***Figure 1*** below).
- Ability to conjugate any protein molecule containing a free amino group to the beads.
- Compatible with a wide selection of flow cytometers for ease of analysis.
- Specific reagents available for confirming success of conjugation reactions.
- Supporting reagents and procedures available for performing instrument setups and assays.

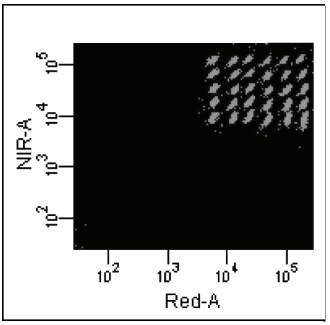

Figure 1

Limitations

- For Research Use Only.
- Not recommended for use on stream-in-air instruments where signal intensities may be reduced. Stream-in-air instruments include the BD FACStar™ Plus and the BD FACSVantage™ flow cytometers.
- Protein must be purified and free of BSA, glycine, Tris, or any other proteins or protein stabilizing additives. The protein must be suspended in PBS, pH 7.2 ± 0.2 before attempting the coupling reaction.
- Not suitable for conjugation of ascites or crude antiserum.
- Although suitable for conjugation of any protein with a free amino group, the protocol has only been validated for IgG subclass antibodies.
- Functional Beads are stable at 4°C for 1 year after conjugation.
- Do not use expired reagents.

Warnings and Precautions

The Storage Buffer contains 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.

Reagents Provided

Store reagents provided undiluted at 4°C.

Coupling Buffer: 1 bottle, 150 mL (1×)

Storage Buffer*: 1 bottle, 90 mL (1×)

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

Equipment and Materials Required but Not Provided

The following items are required for optimal results:

Flow Cytometer

- 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 1 for examples of compatible instrument platforms.

Analytical Balance

- Readability of 0.1 mg (VWR Cat. No. XS205DU) or equivalent

Microcentrifuge

- Microcentrifuge model 5415C (VWR Cat. No. 20901-051) or equivalent

Swing Bucket Centrifuge

- International Equipment Company (IEC/CENTRA CL2 Benchtop) or equivalent

Vortex

- Vortex-Genie Mixers, Scientific Industries (VWR Cat. No. 58816-121) or equivalent

Sonicator

- Table-top ultrasonic cleaner (Branson 1510-DTH or equivalent)

Digital Stirrer

- Eppendorf Thermo R mixer (VWR Cat. No. 21516-166) or equivalent

Spin Columns

- Bio-Spin Columns (Bio-Rad Cat. No. 732-6231)

Chemicals

- DTT, Dithiothreitol (Pierce Cat. No. 20290)
- Sulfo-SMCC, Sulfosuccinimidyl 4-N-maleimidomethyl cyclohexane 1-carboxylate (Pierce Cat. No. 22322)
- NEM, N-Ethylmaleimide (Pierce Cat. No. 23030)
- DMSO, Dimethylsolfoxide (Pierce Cat. No. 20684)

Microcentrifuge Tubes

- Eppendorf Safe-lock Tubes, 1.5 mL (VWR Cat. No. 21008-959) or equivalent

Test Tubes

- 12 x 75 mm, 5 mL Polystyrene Round Bottom Test Tube (BD Cat. No. 352008)

Buffers

- PBS, Dulbecco's Phosphate Buffered Saline 1x (Invitrogen Cat. No. 14040-117)

Supporting Reagents (Recommended)

The following items from BD Biosciences are recommended for optimal results;

Functional Bead Ig Detectors (for validating conjugation)

- PE Goat anti-Mouse Ig Detector, 25 Tests, Cat. No. 558550
- PE Goat anti-Rat Ig Detector, 25 Tests, Cat. No. 558551
- PE Goat anti-Rabbit IgG Detector, 25 Tests, Cat. No. 558553

Assay Buffer Kits (for running assays; choose one depending on protein being assayed)

- Cell Signaling Master Buffer Kit, 100 or 500 Tests, Cat. No. 558223 or 558224
- Human Soluble Protein Master Buffer Kit, 100 or 500 Tests, Cat. No. 558264 or 558265
- Mouse/Rat Soluble Protein Master Buffer Kit, 100 or 500 Tests, Cat. No. 558266 or 558267

Reagents (for validating conjugation with biotinylated protein)

- Streptavidin-PE, Cat. No. 554061

Conjugation Procedure

Before starting the procedure:

1. Bring all provided reagents to room temperature.
2. Remember to protect functional beads from light at all times. Cover tubes with aluminum foil during procedure.
3. Protein sample must be free of BSA, glycine, Tris, or any other proteins or protein stabilizing additives. The protein must be suspended in PBS, pH 7.2 ± 0.2 before attempting the coupling reaction.
   a. The protein sample must be in a buffer that is free of other proteins (eg, BSA or FBS). Purification may be required.
   b. If the protein is in a buffer containing amino groups (eg, Tris or glycine buffer), then a buffer exchange is required. For buffer exchange dialyze overnight in PBS or use a buffer exchange column (recommended Bio-Rad Cat. No. 732-6221).
4. You will need 90 µg of protein for a 500 test conjugation reaction or 180 µg of protein for a 1000 test conjugation reaction. The protein must be at a stock concentration of 1 mg/mL.

   Note: This protocol has not been validated for scalability at anything other than 500 or 1000 tests.

Step I. Bead Preparation

1. Select desired functional beads for conjugation. Vortex functional beads for 30 sec.
2. Transfer the functional beads to a microcentrifuge tube. Cover tube with aluminum foil to protect from light.
   a. 500 test reaction: Transfer 75 µL of functional beads.
   b. 1000 test reaction: Transfer 150 µL of functional beads.
3. Sonicate functional beads for 1 min.
4. Prepare 1M DTT in H_2O

   Note: It is recommended to prepare aliquots of DTT to be stored at –20°C. Use a fresh aliquot for each conjugation.
5. Add 1M DTT to microcentrifuge tube containing the functional beads.
   a. 500 test reaction: Add 1.9 µL of 1 M DTT.
   b. 1000 test reaction: Add 3.8 µL of 1 M DTT.
6. Vortex microcentrifuge tube for 5 sec. Avoid excessive splashing on top of tube.
7. Incubate beads on an orbital shaker for 1 hour at room temperature, protect from light. If a shaker is not available, vortex the beads every 15 min, protect from light.

   Note: Incubation time can be used to prepare the sulfo-SMCC reagent for protein modification (See Step II. #2 below).
8. Add 1 ml of coupling buffer and vortex for 5 sec. (use same volume for 500 and 1000 test reactions).
9. Pellet beads by centrifugation at 900 × *g* for 3 min. Aspirate and discard the supernatant – do not aspirate beads or disturb bead pellet.
10. Repeat steps 8 and 9 three times.
11. Resuspend bead pellet with coupling buffer. The functional beads are ready for coupling with protein.
    a. 500 test reaction: Resuspend with 20 µL of coupling buffer.
    b. 1000 test reaction: Resuspend with 40 µL of coupling buffer.

Step II. Protein Modification

1. Prepare a microcentrifuge tube containing protein stock solution at 1 mg/ml in PBS, pH 7.2 ± 0.2. Cover tube with aluminum foil to protect from light.
   a. 500 test reaction: Prepare a tube with 90 µL protein stock solution.
   b. 1000 test reaction: Prepare a tube with 180 µL protein stock solution.
2. Prepare sulfo-SMCC stock solution (2 mg/mL in DI H_2O) immediately before use.

   Note: Do not store or reuse sulfo-SMCC stock solution.

3. Add sulfo-SMCC to protein stock solution.
   a. 500 test reaction: Add 2 µL of sulfo-SMCC.
   b. 1000 test reaction: Add 4 µL of sulfo-SMCC.
4. Vortex microcentrifuge tube for 5 sec. Avoid excessive splashing on top of tube.
5. Incubate protein on an orbital shaker for 1 hour at room temperature, protect from light. If a shaker is not available, vortex the beads every 15 min, protect from light.

   Note: Incubation time can be used to equilibrate the spin column with Coupling Buffer (See step III. #1 below).

Step III. Buffer Exchange to Remove Unreacted Components

1. To a Bio-Rad Spin Column (Cat. No. 732-6231) add Coupling Buffer until full. Let Coupling Buffer drain from column by gravity, repeat twice (same for 500 and 1000 test reactions).

   Note: This takes approximately 35 min. Refer to the instruction manual from Bio-Rad for detailed instructions.
2. Place the spin column in a 12 × 75 mm test tube and centrifuge the spin column at 1,000 × *g* for 2 min. Discard the 12 × 75 mm tube after centrifugation.
3. Place the spin column in a new 12 × 75 mm test tube.
4. Transfer the entire volume of protein/sulfo-SMCC solution to the spin column.
5. Centrifuge the spin column at 1,000 × *g* for 2 min 15 sec. This transfers the modified protein into the 12 × 75 mm test tube in Coupling Buffer. Discard spin column.
6. It is important to keep the protein/sulfo-SMCC reaction time to one hour as specified in step II. After buffer exchange, proceed immediately to step IV.

Step IV. Protein Conjugation

1. Transfer all the modified protein from the 12 × 75 mm tube to the microcentrifuge tube containing the functional beads previously prepared in Step I.
2. Vortex microcentrifuge tube for 5 sec. Avoid excessive splashing on top of tube.
3. Incubate protein and beads on an orbital shaker for 1 hour at room temperature, protect from light. If a shaker is not available, vortex the beads every 15 min, protect from light.
4. Prepare NEM (2 mg/ml in DMSO).

   Note: It is recommended to prepare 20 µl aliquots of NEM solution and store at -20°C. Use a fresh aliquot for each conjugation.

5. Add NEM solution to the microcentrifuge tube containing the functional beads and modified protein.
   a. 500 test reaction: Add 2 µL of NEM.
   b. 1000 test reaction: Add 4 µL of NEM.
6. Vortex microcentrifuge tube for 5 sec. Avoid excessive splashing on top of tube.
7. Incubate microcentrifuge tube for 15 min on an orbital shaker. If a shaker is not available, vortex periodically during the incubation.
8. Add 1 ml of Storage Buffer to the microcentrifuge tube (Use same volume for 500 and 1000 test reactions).
9. Pellet beads by centrifugation at 900 × *g* for 3 min. Aspirate and discard the supernatant – do not aspirate beads or disturb bead pellet.
10. Repeat steps 8 and 9 three more times.
11. Resuspend bead pellet in Storage Buffer. The beads are now at a final concentration of approximately 6×10^6 beads/ml and are ready to be validated.
    a. 500 test reaction: Resuspend pellet in 0.5 mL of Storage Buffer.
    b. 1000 test reaction: Resuspend pellet in 1.0 mL of Storage Buffer.

    Note: Store beads at 4°C and protect from light. For optimal results let beads sit in storage buffer overnight prior to first use. The background of the beads will decrease slightly overnight. The beads are stable for 1 year after conjugation.

Conjugation Confirmation

Once the beads are conjugated it is recommended to confirm the success of the conjugation procedure before using the conjugated beads in an assay. This confirmation can be performed using a flow cytometer (Refer to ***Appendix***, table 1 for examples of compatible instrument platforms) and a phycoerythrin (PE) conjugated molecule that binds specifically with the protein conjugated to the bead. Depending on the reagents used, a successful conjugation normally gives a PE signal greater than 500 MFI. Below are two suggested methods for determining a successful conjugation.

1. To test for antibodies that have been conjugated to the functional beads, BD Biosciences offers PE anti-Ig Detectors (See ***Supporting Reagents*** for more information). Ensure the correct reagent is used to confirm conjugation by matching the species of the antibody that was conjugated to the functional beads with the PE anti-Ig Detector (eg, mouse, rat, rabbit).

   Note: See the Functional Bead Ig Detectors Procedure below for the Conjugation Confirmation protocol using a Functional Bead Ig Detector.
2. To test for non-antibody proteins that have been conjugated to the functional beads:
   a. Use a PE-labeled antibody that is specific to that protein.
   b. Use a biotinylated antibody specific for that protein followed by streptavidin-PE.

Functional Bead Ig Detector Procedure

Before starting the procedure:

1. Bring all provided reagents to room temperature.
2. Remember to protect functional beads from light at all times. Cover tubes with aluminum foil during procedure.

Procedure

1. Label three 12 × 75 mm test tubes, *(1) diluted functional beads, (2) negative control, and (3) test sample.*
2. Vortex the bulk conjugated functional beads for 5 sec.
3. To the diluted functional beads tube (1) add 245 µL of Wash Buffer from one Master Buffer Kit used to run the assay (see ***Supporting Reagents*** section for more information).

   Note: Do not use Capture Bead Diluent from Master Buffer Kit to dilute beads when confirming conjugation.
4. Add 5 µL of the bulk functional beads to the *diluted functional beads* tube (1) and vortex the diluted functional beads for 5 sec.
5. Transfer 50 µL of the beads from the *diluted functional beads* tube (1) to the *negative control* tube (2) and to the *test sample* tube (3). The *diluted functional beads* tube (1) can now be discarded.
6. Add 50 µL of Wash Buffer to the *negative control* tube (2).
7. Add 50 µL of the appropriate PE anti-Ig Detector to the *test sample* tube (3).
8. Vortex *negative* and *test sample* control tubes for 5 sec.
9. Incubate tubes for 30 minutes in the dark at room temperature.
10. Add 1 mL of wash buffer to both tubes.
11. Centrifuge the tubes at 1,000 × g for 5 min. Aspirate and discard the supernatant – do not aspirate beads or disturb bead pellet.
12. Re-suspend bead pellets for both tubes with 150 µL of Wash Buffer.
13. Read both tubes on a flow cytometer (Refer to ***Appendix***, table 1 for examples of compatible instrument platforms.) using the appropriate BD CBA Flex Setup instrument settings (Refer to ***BD™ CBA Instrument Setup manual*** for instructions).
14. For flow cytometers that have plate readers, transfer beads into two free wells on a 96 well plate and read using the appropriate BD CBA Flex Setup instrument settings (Refer to ***BD CBA Instrument Setup manual*** for instructions).
15. If the signal for the test sample is 500 MFI greater than the signal for the negative control sample then the conjugation was successful and the conjugated beads can be used in further assays.

    Note: This procedure will give a qualitative result but it can be used to get an idea of how much antibody was conjugated to the functional bead. If there is no signal difference between the negative control and the test sample then no antibody was successfully conjugated.

Troubleshooting Tips

Problem	Possible Causes	Suggested Solution
Low bead number after conjugation	Inadequate vortexing	Vortex beads for given amount of time in procedure. Beads can aggregate.
	Bead loss during aspiration steps	Carefully aspirate supernatant only during wash steps.
Low to no signal when confirming conjugation	Buffers or chemicals may have expired	Check espiration dates on all reagents.
	Sulfo-SMCC stock solution was not made immediately before use	Prepare Sulfo-SMCC during conjugation procedure. Do not store or reuse.
	Wrong molecule was used to detect conjugation	Ensure molecule recognizes protein conjugated to bead. If antibody was conjugated, check species of antibody to ensure the correct detector was used.
	Protein sample is not purified	Make sure the protein is free of BSA, glycine, Tris, or any other proteins or protein stabilizing additives.
	Wrong buffer used to suspend protein	The protein needs to be suspended in PBS.
	The protein should be concentrated to 1 mg/mL	If the protein concentration is lower, then there is the possibility of less protein conjugating to the bead.
	Capture Bead Bottling buffer used to dilute functional beads	Use Wash Buffer from Master Buffer Kit to dilute functional beads.
Low to no signal in assay	Functional bead conjugation failed	Confirm conjugation.
	Protein conjugated to bead does not recognize desired analyte	Conjugate desired analyte with PE or Biotin followed by streptavidin-PE. Using a flow cytometer, see if bead can recognize PE conjugated analyte.
	Antibody pair does not work in CBA format	Check if antibody pair works by ELISA. Try different antibodies.
	Antibody pair does not recognize different epitopes of the protein	Check if antibody pair works by ELISA.
Assay recognizes recombinant but not native protein	Wrong immunogenic material used to develop antibodies	Check integrity of recombinant protein .
Assay standard curve is not parallel to native sample curve	Wrong immunogenic material used to develop antibodies	Check integrity of recombinant protein .

Immunoassay Development Hints

Identifying an antibody pair

To develop a bead-based immunoassay, often a pair of antibodies is needed. That means having two antibodies that bind to the same molecule at the same time, therefore recognizing different epitopes. One antibody will be conjugated to the functional bead and the other antibody will be used as the detection antibody to detect the presence of the analyte of interest bound to the conjugated bead. A good starting point is to use a pair of antibodies that work in an ELISA.

It is difficult to predict which antibody out of the pair will function best as the capture antibody. If a known ELISA pair is being used, generally the capture antibody for the ELISA works best as the capture antibody with the functional beads. However, this is not always the case. If there is enough material, it is best to try both antibodies as capture and detector.

Confirming the bead conjugation

After the antibody has been conjugated to the beads, you should test to see if the reaction was successful. This can be accomplished by performing the Functional Bead Ig Detector Procedure using the appropriate PE anti-Ig Detector (See ***Supporting Reagents*** for more information).

When validating conjugation or running an assay it is recommended to use the detection fluorochrome. PE can either be directly conjugated to the detection protein or a biotinylated protein can be used followed by streptavidin-PE. PE is chosen because of its brightness, which results in a broad dynamic range. Fluorochromes that emit in the red spectrum should not be used due to spectral overlap with the beads.

Confirming that the new assay measures the correct analyte

The conjugated functional beads and detector need to be tested against a standard, such as a recombinant protein, and against samples that contain the native protein. The samples can be tissue culture supernatant, serum, plasma or any other good source for the native protein of interest. For the measurement of intracellular proteins, detergent lysates of cells should be used.

It is important that your assay measures the particular molecule of interest in the same range that occurs physiologically. For most cytokine assays, the likely range of detection will be from 5000 pg/ml down to 20 pg/ml or lower.

Evaluating linearity of the assay

Another important consideration is the linearity of the standard curve. In particular, the standard curve and the sample titration curve must be parallel to each other. This will ensure that values read off the top end of the standard curve will be comparable to values read off of the low end. If the curves are not parallel, the results obtained for sample dilutions (taking into account the dilution factor) will not be the same across the standard curve range.

Optimizing the assay

If the signal is low, consider the following:

a. One possible solution, as mentioned above is to switch the pairs. It has been seen in a number of cases that the best ELISA capture antibody may not work well as the BD™ CBA capture antibody.

b. The timing of the assay can be altered. An assay can be 1-step in which beads, sample, and detector are all added at the same time or it can be a 2-step assay in which beads and sample are pre-incubated before the addition of detector. In the case of a 2-step assay, it may be beneficial to wash before the addition of detector.

c. The timing of the various steps can also influence the total signal that is observed.

d. The detector concentration should be optimized. Generally, initial studies can be done using 200 ng of detector antibody per test. An experiment should be done in which the detector concentration is titrated down. An example would be to run a standard curve and samples and use detector concentrations from 12.5 ng/test up to 200 ng/test. Using higher detector concentrations than are necessary will use up the reagent more quickly and, more importantly, can lead to higher backgrounds.

Multiplexing the assay with existing BD™ CBA Flex Set assays

If the new bead-based immunoassay is to be mixed with any existing BD™ CBA Flex Set assay, then experiments must be performed to determine compatibility of the assays. The new bead-based immunoassay must use the same assay procedure and buffers as the BD CBA Flex Set assay it is to be mixed with. It is critical to confirm that the new bead-based assay and the BD CBA Flex Set assay perform the same way as a single bead as they do in a mixture. A poor correlation between the two is usually due to cross-reactivity or non-specific binding. Look not only at how the other assays affect your assay but also how your new combination affects the existing bead sets. The following tests are recommended:

1. Make a mixture of all of the beads and all of the detectors. The standards for each assay are then added individually to different wells. A signal should only be seen with the correct bead.
2. A standard curve and a titration of a positive test sample should be tested with a single bead assay and with a multiplex of bead assays. The quantitation of the test sample should be the same regardless of which other assays are being run with it.

   Note: If either test gives results that are unexpected, it is likely that the assays can not be mixed and should be run separately in the future.

Appendix

Table 1. Recommended Instrument Platforms

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

Notes

Notes

Notes

United States
877.232.8995

Canada
888.259.0187

Europe
32.53.720.550

Japan
0120.8555.90

Asia/Pacific
65.6861.0633

Latin America/Caribbean
55.11.5185.9645

BD Biosciences
10975 Torreyana Road
San Diego, CA 92121
Customer/Technical Service
Tel 877.232.8995 (US)
Fax 800.325.9637
bdbiosciences.com

06-790030-558556A