BD Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit

Instruction Manual

Catalog No. 560485

BD

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History

Revision	Date	Change Made
647209	2/09	New document

BD flow cytometers are class I (1) laser products.

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Contents

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1

About this kit

This section covers the following topics:

Purpose of the kit

About this topic

This topic explains the purpose of the BD™ Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine Kit (Catalog No. 560485), and provides background for understanding the kit's components and how they work.

Use of the kit

The BD CBA Mouse Th1/Th2/Th17 Cytokine Kit can be used to measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interferon-γ (IFN-γ), Tumor Necrosis Factor (TNF), Interleukin-17A (IL-17A), and Interleukin-10 (IL-10) protein levels in a single sample. The kit performance has been optimized for analysis of physiologically relevant concentrations (pg/mL levels) of specific cytokine proteins in tissue culture supernatants and serum samples. The kit provides sufficient reagents for the quantitative analysis of 60 samples and the generation of two standard curve sets.

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 a BD CBA kit has been conjugated with a specific antibody. The detection reagent provided in the kit is a mixture of phycoerythrin (PE)–conjugated antibodies, which provides a fluorescent signal in proportion to the amount of bound analyte.

When the capture beads and detector reagent are incubated with an unknown sample 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.

Principle of this assay

The BD CBA Mouse Th1/Th2/Th17 Cytokine Kit uses bead array technology to simultaneously detect multiple cytokine proteins in research samples. Seven bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A, and IL-10 proteins. The seven bead populations are mixed together to form the bead array, which is resolved in a red channel (ie, FL3 or FL4) of a flow cytometer.

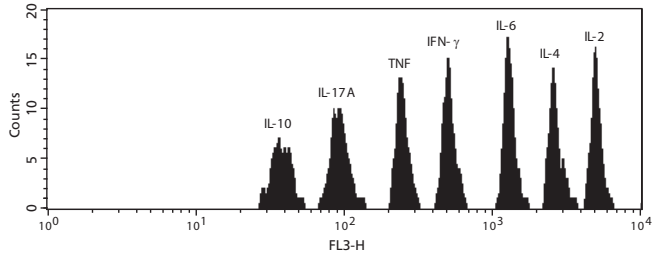

Figure 1

During the assay procedure, you will mix the cytokine capture beads with recombinant standards or unknown samples and incubate them with the PE-conjugated detection antibodies to form sandwich complexes. The intensity of PE fluorescence of each sandwich complex reveals the concentration of that cytokine. After acquiring samples on a flow cytometer, use FCAP Array™ software to generate results in graphical and tabular format.

Advantages over ELISA

The broad dynamic range of fluorescent detection via flow cytometry and the efficient capturing of analytes via suspended particles enable the BD CBA assay to measure the concentration of an unknown in substantially less time and using fewer sample dilutions compared to conventional ELISA methodology.

- The required sample volume is approximately one-seventh the quantity necessary for conventional ELISA assays due to the detection of seven analytes in a single sample.
- A single set of diluted standards is used to generate a standard curve for each analyte.
- A BD CBA experiment takes less time than a single ELISA and provides results that would normally require seven conventional ELISAs.

Related topics

- Limitations (page 4)
- Kit contents (page 5)

Limitations

About this topic

This topic lists the limitations of the assay.

Limitations

The theoretical limit of detection of the BD CBA Mouse Th1/Th2/Th17 Cytokine Kit is comparable to conventional ELISA, but due to the complexity and kinetics of this multi-analyte assay, the actual limit of detection on a given experiment may vary. See Theoretical limit of detection (page 36) and Precision (page 41).

The BD CBA is not recommended for use on stream-in-air instruments where signal intensities may be reduced, adversely effecting assay sensitivity. Stream-in-air instruments include the BD FACStar™ Plus and BD FACSVantage™ (BD Biosciences, San Jose, CA) flow cytometers.

Serum spike recoveries for IL-4 and TNF are lower than for the other cytokines in this assay. This variation is due to assay conditions and serum proteins and may affect quantitation of these proteins in serum samples.

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

This kit is designed to be used as an integral unit. Do not mix components from different batches or kits.

Related topics

- Purpose of the kit (page 2)

Kit contents

About this topic

This topic describes the contents of the Mouse Th1/Th2/Th17 Cytokine Kit.

Contents

The Mouse Th1/Th2/Th17 Cytokine Kit contains the following components sufficient for 80 tests (60 samples and two standard curves):

Vial label	Reagent	Quantity
A1	Mouse IL-2 Capture Beads	1 vial, 0.8 mL
A2	Mouse IL-4 Capture Beads	1 vial, 0.8 mL
A3	Mouse IL-6 Capture Beads	1 vial, 0.8 mL
A4	Mouse IFN-γ Capture Beads	1 vial, 0.8 mL

Vial label	Reagent	Quantity
A5	Mouse TNF Capture Beads	1 vial, 0.8 mL
A6	Mouse IL-17A Capture Beads	1 vial, 0.8 mL
A7	Mouse IL-10 Capture Beads	1 vial, 0.8 mL
B	Mouse Th1/Th2/Th17 PE Detection Reagent	1 vial, 4 mL
C	Mouse Th1/Th2/Th17 Cytokine Standards	2 vials, 0.2 mL lyophilized
D	Cytometer Setup Beads	1 vial, 1.5 mL
E1	PE Positive Control Detector	1 vial, 0.5 mL
E2	FITC Positive Control Detector	1 vial, 0.5 mL
F	Wash Buffer	1 bottle, 130 mL
G	Assay Diluent	1 bottle, 30 mL

Bead reagents

Mouse Cytokine Capture Beads (A1–A7): A single 80-test vial of each specific capture bead (A1–A7). The specific capture beads, having discrete fluorescence intensity characteristics, are distributed from brightest (A1) to dimmest (A7).

Cytometer Setup Beads (D): A single 30-test vial of setup beads for setting the initial instrument PMT voltages and compensation settings is sufficient for 10 instrument setup procedures. The Cytometer Setup Beads are formulated for use at 50 µL/test.

Antibody and standard reagents

Mouse Th1/Th2/Th17 PE Detection Reagent (B): An 80-test vial of PE-conjugated anti-mouse IL-2, IL-4, IL-6, IFNγ, TNF, IL-17A, and IL-10 antibodies that is formulated for use at 50 µL/test.

Mouse Th1/Th2/Th17 Cytokine Standards (C): Two vials containing lyophilized recombinant mouse cytokine proteins. Each vial should be reconstituted in 2.0 mL of Assay Diluent to prepare the top standard.

PE Positive Control Detector (E1): A 10-test vial of PE-conjugated antibody control that is formulated for use at 50 µL/test. This reagent is used with the Cytometer Setup Beads to set the initial instrument compensation settings.

FITC Positive Control Detector (E2): A 10-test vial of FITC-conjugated antibody control that is formulated for use at 50 µL/test. This reagent is used with the Cytometer Setup Beads to set the initial instrument compensation settings.

Buffer reagents

Wash Buffer (F): A single 130-mL bottle of phosphate buffered saline (PBS) solution (1×), containing protein and detergent, used for wash steps and to resuspend the washed beads for analysis.

Assay Diluent (G): A single 30-mL bottle of a buffered protein solution (1×) used to reconstitute and dilute the Mouse Th1/Th2/Th17 Cytokine Standards and to dilute unknown samples.

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

Related topics

- Storage and handling (page 8)

Storage and handling

About this topic

This topic describes the requirements for kit storage and handling, and includes a warning for hazardous ingredients.

Storage

Store all kit components at 4°C. Do not freeze.

Warning

Components A1–A7, B, D, E1, E2, F, and G 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:

Assay workflow

About this topic

This topic provides an overview of all the steps necessary to perform the assay.

Workflow

The overall workflow consists of the following steps:

Step	Description
1	Preparing Mouse Th1/Th2/Th17 Cytokine Standards (page 14)
2	Mixing Mouse Th1/Th2/Th17 Cytokine Capture Beads (page 16)
3	Diluting samples (page 17), if necessary
4	Performing setup with Cytometer Setup Beads (page 21) **Note:** Can be performed during step 5 incubation.
5	Staining Mouse Th1/Th2/Th17 Cytokine samples (page 28)
6	Acquiring samples (page 30)
7	Data Analysis (page 33)

Incubation times

To help you plan your work, the incubation times are listed in the following table:

Procedure	Incubation time
Preparing standards	15 minutes
Preparing Cytometer Setup Beads	30 minutes
Staining samples for analysis	2 hours

Required materials

About this topic

This topic describes the reagents, consumables, and equipment you will need to use with the kit for analyzing samples for the relevant concentrations of specific cytokines.

Materials required but not provided

In addition to the reagents provided in the BD CBA Mouse Th1/Th2/Th17 Cytokine Kit, the following items are also required:

- A flow cytometer capable of detecting and distinguishing fluorescence emissions at 576 and 670 nm (eg, BD FACScan™, BD FACSCalibur™, BD FACSArray™, BD FACSCanto™ II, BD™ LSR II, or BD FACSAria™ II flow cytometer) and BD CellQuest™ or BD CellQuest Pro software

  Note: This manual describes setup for BD FACScan and BD FACSCalibur flow cytometers only.

- BD Falcon™ 12 × 75-mm sample acquisition tubes (Catalog No. 352008), or equivalent
- 15-mL conical, polypropylene tubes (BD Falcon, Catalog No. 352097), or equivalent
- FCAP Array software (Catalog No. 641488 [PC] or 645447 [Mac])
- BD Calibrite™ 3 beads (Catalog No. 340486)
- BD Calibrite APC beads (Catalog No. 340487), for dual-laser BD FACSCalibur instruments

Related topics

- Kit contents (page 5)

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Assay preparation

This section covers the following topics:

Preparing Mouse Th1/Th2/Th17 Cytokine Standards

About this topic

This topic describes how to reconstitute and serially dilute the Mouse Th1/Th2/Th17 Cytokine Standards.

Purpose of this procedure

The Mouse Th1/Th2/Th17 Cytokine Standards are lyophilized and should be reconstituted and serially diluted immediately before mixing with the Capture Beads and the PE Detection Reagent.

You must run the cytokine standards in each experiment. Do not store or reuse reconstituted or diluted standards.

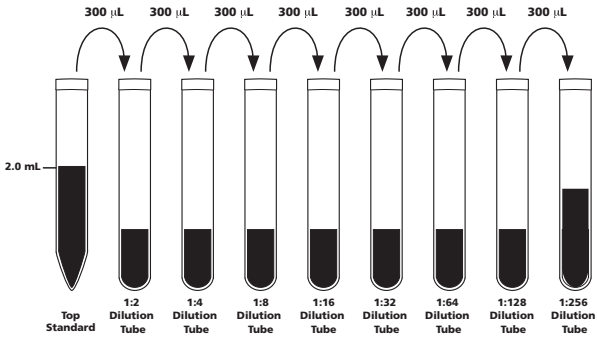
Procedure

To reconstitute and serially dilute the standards:

1. Open one vial of lyophilized Mouse Th1/Th2/Th17 Standards. Transfer the standard spheres to a 15-mL conical, polypropylene tube. Label the tube "Top Standard."
2. Reconstitute the standards with 2.0 mL of Assay Diluent.
    a. Allow the reconstituted standard to equilibrate for at least 15 minutes at room temperature.
    b. Gently mix the reconstituted protein by pipette only. Do not vortex or mix vigorously.
3. Label 12 × 75-mm tubes and arrange them in the following order: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256.
4. Pipette 300 µL of Assay Diluent in each of the 12 × 75-mm tubes.
5. Perform serial dilutions:
    a. Transfer 300 µL from the Top Standard to the 1:2 dilution tube and mix thoroughly by pipette only.

b. Continue making serial dilutions by transferring 300 µL from the 1:2 tube to the 1:4 tube and so on to the 1:256 tube.

c. Mix thoroughly by pipette only. Do not vortex.



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

Concentration of standards

See the Procedure section of Staining Mouse Th1/Th2/Th17 Cytokine samples (page 28) for a listing of the concentrations (pg/mL) of all seven recombinant proteins in each standard dilution.

Next step

Proceed to Mixing Mouse Th1/Th2/Th17 Cytokine Capture Beads (page 16).

Related topics

- Kit contents (page 5)
- Required materials (page 11)

Mixing Mouse Th1/Th2/Th17 Cytokine Capture Beads

About this topic

This topic provides instructions on how to mix the Cytokine Capture Beads prior to using them in the assay.

The Capture Beads are bottled individually (A1–A7). You must pool all seven bead reagents immediately before using them in the assay.

Procedure

To mix the Capture Beads:

1. Determine the number of assay tubes (including standards and controls) required for the experiment (eg, 8 unknowns, 9 cytokine standard dilutions, and 1 negative control = 18 assay tubes).
2. Vigorously vortex each Capture Bead suspension for 3 to 5 seconds before mixing.

   Note: The antibody-conjugated beads will settle out of suspension over time. It is necessary to vortex the vial before taking a bead-suspension aliquot.
3. Add a 10-µL aliquot of each Capture Bead, for each assay tube to be analyzed, into a single tube labeled "mixed Capture Beads" (eg, 10 µL of IL-2 Capture Beads × 18 assay tubes = 180 µL of IL-2 Capture Beads required).
4. Vortex the bead mixture thoroughly.

Next step

The mixed Capture Beads are now ready to be transferred to the assay tubes. Discard excess mixed Capture Beads. Do not store after mixing.

To begin the assay, proceed to Staining Mouse Th1/Th2/Th17 Cytokine samples (page 28). If you need to dilute samples having a high cytokine concentration, proceed to Diluting samples (page 17).

Diluting samples

About this topic

This topic provides instructions on how to dilute tissue culture supernatants and serum samples having a known high cytokine concentration. This procedure is not necessary for all samples.

Purpose of this procedure

The standard curve for each cytokine covers a defined set of concentrations from 20 to 5000 pg/mL. It might be necessary to dilute samples to ensure that their mean fluorescence values fall within the range of the generated cytokine standard curve. For best results, dilute samples that are known or assumed to contain high levels of a given cytokine.

Procedure

To dilute samples with a known high cytokine concentration:

1. Dilute the sample by the desired dilution factor (ie, 1:2, 1:10, or 1:100) using the appropriate volume of Assay Diluent.
2. Mix sample dilutions thoroughly.

Next step

Proceed to Performing setup with Cytometer Setup Beads (page 21). Or, if you wish to begin staining your samples for the assay, proceed to Staining Mouse Th1/Th2/Th17 Cytokine samples (page 28), and you can perform instrument setup during the 2-hour staining incubation.

4

Cytometer setup

This section covers the following topics:

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Requirements for assay setup

About this topic

This topic describes what you need to do and be aware of before preparing and running the Cytometer Setup Beads.

The setup procedure described in this manual applies specifically to the BD FACScan and BD FACSCalibur flow cytometers.

Materials required

Before performing setup, ensure you have the following:

- BD FACSComp software with BD Calibrite beads for daily setup
- BD CellQuest or BD CellQuest Pro software for running the Cytometer Setup Beads
- Cytometer Setup Beads
- FITC and PE Positive Control Detectors
- The appropriate setup template:
  - For single-laser cytometers – BD CBA Instrument Setup template, which can be found on the BD FACStation™ CD for Macintosh computers in the BD CBA folder, or downloaded from bdbiosciences.com/cbatemplates
  - For dual-laser cytometers – BD CBA Dual-Laser Instrument Setup template, which can be downloaded from bdbiosciences.com/cbatemplates

Steps required

If you are using a BD FACScan or BD FACSCalibur flow cytometer, perform daily instrument setup using BD FACSComp software and BD Calibrite beads. Run the BD Calibrite beads in lyse/no-wash mode.

If you are using a dual-laser BD FACSCalibur cytometer, ensure that the second laser is turned on.

The data will be evaluated in five parameters (FSC, SSC, FL1, FL2, and FL3 for single-laser instruments and FSC, SSC, FL1, FL2, and FL4 for dual-laser instruments). Turn off additional detectors.

More information

For information on using BD FACSComp software with BD Calibrite beads, see the *BD FACSComp Software Reference Manual* and the *BD Calibrite Beads* package insert.

Additional setup protocols for the BD FACSArray bioanalyzer and digital BD FACS brand flow cytometers can be found at bdbiosciences.com/cbasetup.

Performing setup with Cytometer Setup Beads

About this topic

This topic describes how to prepare and run the Cytometer Setup Beads to set up the cytometer in preparation for running the assay.

If this is your first time running the Cytometer Setup Beads, review the information in Requirements for assay setup (page 20).

Purpose of the beads

If you are using a single-laser cytometer, three tubes of setup beads are required—tubes A, B, and C. If you are using a dual-laser BD FACSCalibur cytometer, only tube A is required. Tube A allows you to adjust the PMT voltages, whiles tubes B and C allow you to adjust compensation.

Preparing the Cytometer Setup Beads

To prepare the Cytometer Setup Beads:

1. Add 50 µL of Cytometer Setup Beads to three cytometer setup tubes labeled A, B, and C.
2. Add 50 µL of FITC Positive Control Detector to tube B.
3. Add 50 µL of PE Positive Control Detector to tube C.
4. Incubate tubes A, B, and C for 30 minutes at room temperature, protected from light.
5. Add 450 µL of Wash Buffer to tube A and 400 µL of Wash Buffer to tubes B and C.

Adjusting the cytometer before running the beads

Before running the beads, make the following instrument adjustments:

1. Start BD CellQuest or BD CellQuest Pro software and open the appropriate instrument setup template. See Materials required (page 20).
2. Set the instrument to Acquisition mode.
3. Set FSC and SSC to Log mode.
4. Decrease the SSC PMT voltage by 100 from what BD FACSComp software set.
5. Set the Threshold to FSC at 650.
6. **For dual-laser cytometer users only,** set all compensation values to 0.0%.

Any compensation above 0 might adversely affect the performance of the Mouse Th1/Th2/Th17 Cytokine Kit when using the dual-laser protocol.

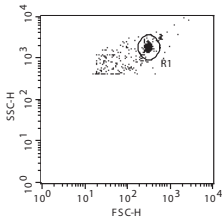
Running the Cytometer Setup Beads

Regardless of whether you are using a single- or dual-laser cytometer, run the Cytometer Setup Beads as follows:

1. Place tube A on the cytometer. Run the beads in setup mode.

   Pause and restart acquisition frequently during the setup procedure to reset detected values after settings adjustments.

2. Adjust gate R1 so that the singlet bead population is located in the gate.



3. Make the following adjustments:

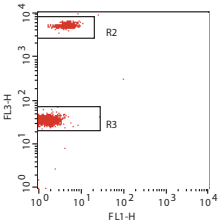
   Adjust the FL3 (or FL4 for dual-laser cytometer) PMT voltage so that the median of the top FL3 (FL4 for dual laser) bead population's intensity is approximately 5000.

   Adjust gate R3 as necessary so that the dim FL3 (or FL4 for dual-laser cytometer) bead population is located in gate R3. Do not adjust the R2 gate.

Adjust the FL1 PMT voltage so that the median of FL1 is approximately 2.0 to 2.5.

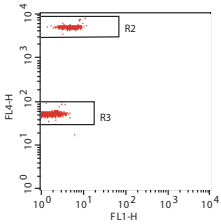
Bright Beads (R2) Median: 5139.70

FL1 Median: 2.53



Single laser

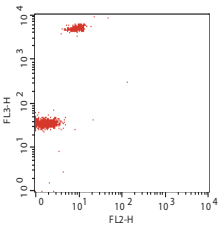
FL1 Median: 2.19



Dual laser

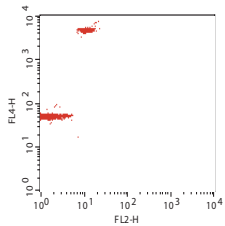
4. Adjust the FL2 PMT voltage so that the median of FL2 is approximately 2.0 to 2.5.
5. Depending on your cytometer:
   - Dual-laser cytometer users save and print optimized instrument settings. Proceed to Staining Mouse Th1/Th2/Th17 Cytokine samples (page 28).
   - Single-laser cytometer users continue with Adjusting compensation (page 25).

FL2 Median: 2.31



Single laser

FL2 Median: 2.21



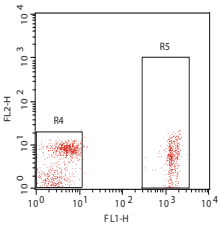
Dual laser

Adjusting compensation

Single-laser cytometer users continue with the remainder of the setup as follows:

1. Place tube B on the cytometer to adjust the FL2–%FL1 compensation setting.
2. Adjust gate R5 as necessary so that the FL1 bright bead population is located in gate R5.

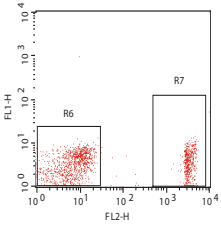
   Adjust the FL2–%FL1 compensation setting so that the median of R5 is equal to the median of R4.



R4 Median 2.94 R5 Median 3.75

3. Place tube C on the cytometer to adjust the FL1–%FL2 and FL3–%FL2 compensation settings.
4. Adjust gate R7 so that the FL2 bright bead population is located in gate R7.

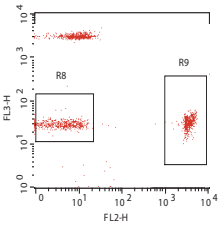
   Adjust the FL1–%FL2 compensation setting so that the median of R7 is equal to the median of R6.



R6 Median 2.81 R7 Median 3.08

5. Adjust gate R9 so that the FL2 bright bead population is located in gate R9.

Adjust the FL3–%FL2 compensation setting so that the median of R9 is equal to the median of R8.



R8 Median 27.88 R9 Median 28.13

6. Save and print the optimized instrument settings.

Next step

Proceed to Staining Mouse Th1/Th2/Th17 Cytokine samples (page 28).

5

Assay procedure

This section covers the following topics:

Staining Mouse Th1/Th2/Th17 Cytokine samples

About this topic

This topic provides instructions for staining both standards and unknown samples to be analyzed.

Before you begin

- Prepare the standards as described in Preparing Mouse Th1/Th2/Th17 Cytokine Standards (page 14).
- Mix the Capture Beads as described in Mixing Mouse Th1/Th2/Th17 Cytokine Capture Beads (page 16).
- If necessary, dilute the unknown samples. See Diluting samples (page 17).

Procedure

To prepare the standards and samples for analysis:

1. Vortex the mixed Capture Beads and add 50 µL to all assay tubes.
2. Add 50 µL of the Mouse Th1/Th2/Th17 Cytokine Standard dilutions to the control tubes as listed in the following table:

Tube label	Concentration (pg/mL)	Cytokine Standard dilution
1	0 (negative control)	no standard dilution (Assay Diluent only)
2	20	1:256
3	40	1:128
4	80	1:64
5	156	1:32
6	312.5	1:16
7	625	1:8

Tube label	Concentration (pg/mL)	Cytokine Standard dilution
8	1250	1:4
9	2500	1:2
10	5000	Top standard

3. Add 50 µL of each unknown sample to the appropriately labeled sample assay tubes.
4. Add 50 µL of the Mouse Th1/Th2/Th17 PE Detection Reagent to all assay tubes.
5. Incubate the assay tubes for 2 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. See Cytometer setup (page 19).

6. Add 1 mL of Wash Buffer to each assay tube and centrifuge at 200*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 to resuspend the bead pellet.

Next step

Proceed to Acquiring samples (page 30).

It is necessary to acquire BD CBA 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.

Acquiring samples

About this topic

This topic describes the steps for acquiring samples using BD CellQuest software. If you are using a BD FACSArray bioanalyzer or digital BD FACS brand cytometer, see that cytometer's user's guide for specific details.

Before you begin

Run the assay setup procedure. See Performing setup with Cytometer Setup Beads (page 21) for information.

Prepare samples for analysis. See Staining Mouse Th1/Th2/Th17 Cytokine samples (page 28).

Vortex each sample for 3 to 5 seconds immediately before acquiring on the flow cytometer.

Ensure the appropriate acquisition template is available. It can be downloaded from bdbiosciences.com/cbatemplates.

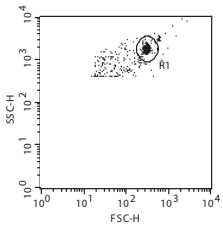
Procedure

To acquire samples:

1. Open the appropriate acquisition template.
2. Set acquisition mode and retrieve the optimized instrument settings from Performing setup with Cytometer Setup Beads (page 21).
3. In the Acquisition and Storage window, set the resolution to 1024.
4. Set the number of events to be counted to 2100 of R1 gated events. This ensures that the sample file contains approximately 300 events per Capture Bead.
5. Set the number of events to be collected to "all events." Saving all events collected ensures that no true bead events are lost due to incorrect gating.

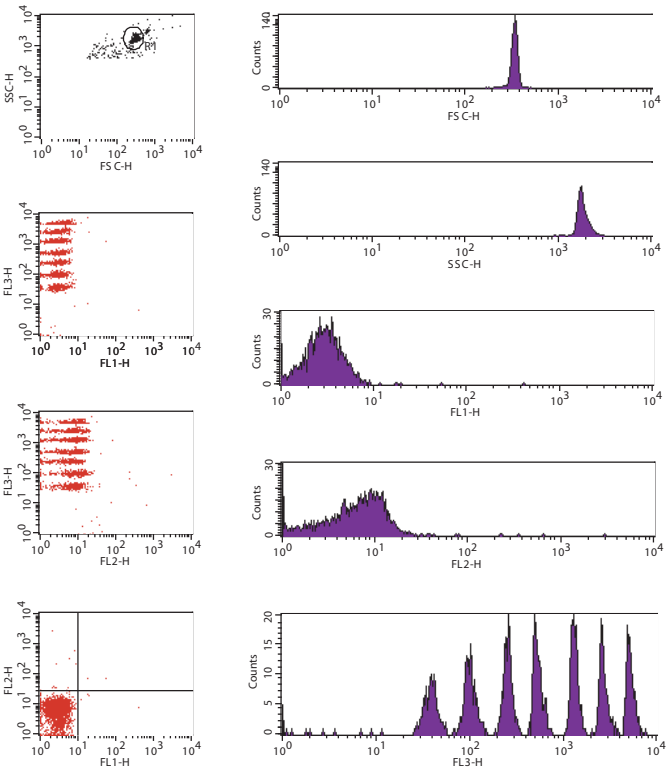
6. If you are running a dual-laser instrument, click the **Parameters Saved** button. Ensure that only FSC-H, SSC-H, FL1-H, FL2-H, and FL4-H are selected. It is important that additional detectors be turned off. Click **OK.**

7. Vortex tube 1 (negative control) for 3 to 5 seconds. Run the tube in setup mode. Using the FSC vs SSC dot plot, place the R1 region gate around the singlet bead population.



8. Specify a file name. Ensure that the file name is alphanumeric. FCAP Array software requires file names containing some alpha characters.

9. Begin sample acquisition with the flow rate set at HIGH. See the following example acquisition template.



10. Continue acquiring samples. Vortex each tube for 3 to 5 seconds before acquiring. Run tube 2 (20 pg/mL), followed by tube 3 (40 pg/mL), and so on through tube 10 (Top Standard). Run the unknown samples after the standards.

Next step Proceed to Data Analysis (page 33).

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Data Analysis

About this topic

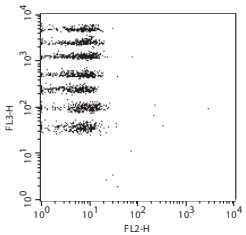
This topic shows examples of Mouse Th1/Th2/Th17 Cytokine data acquired using BD CellQuest software, and specifies the software required to analyze the data.

How to analyze

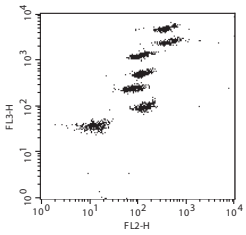
Analyze Mouse Th1/Th2/Th17 Cytokine data using FCAP Array software. For instructions on using FCAP Array software, go to bdbiosciences.com/docs/FCAP_Array_analysis_of_CBA_Kits.pdf.

Typical data

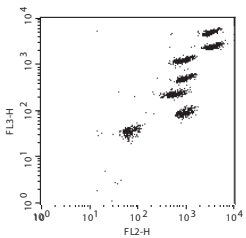
The following data, acquired using BD CellQuest software, shows standards and detectors alone.



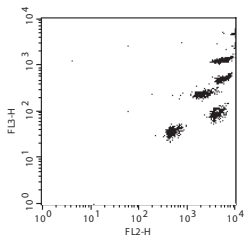
Negative control (0 pg/mL)



Standard 80 pg/mL



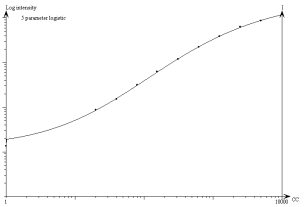
Standard 625 pg/mL



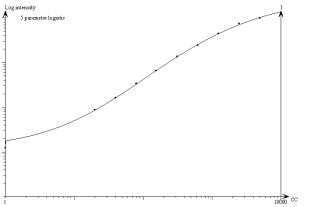
Standard 5000 pg/mL

Standard curve examples

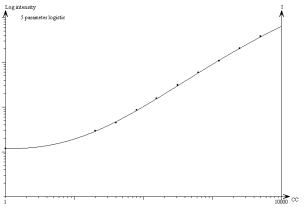
The following graphs represent standard curves from the Mouse Th1/Th2/Th17 Cytokine Standards.



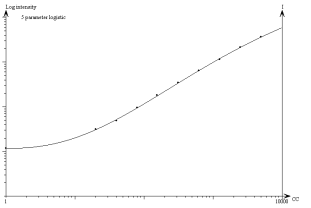
IL-2



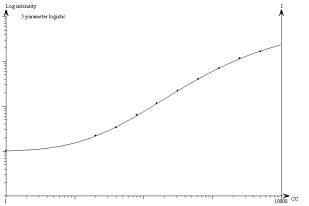
IL-4



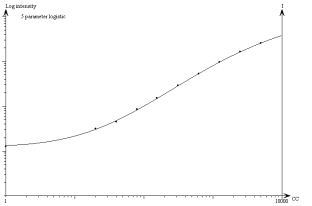
IL-6



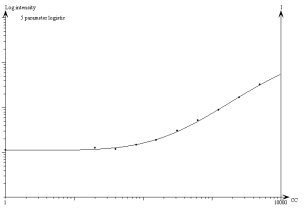
IFN-γ



TNF



IL-17A



IL-10

6

Performance

This section covers the following topics:

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Theoretical limit of detection

About this topic

This topic describes the principle and results of the theoretical limit of detection experiment.

How performed

The individual standard curve range for a given cytokine defines the minimum and maximum quantifiable levels using the Mouse Th1/Th2/Th17 Cytokine Kit (ie, 20 pg/mL and 5000 pg/mL). By applying the 4-parameter curve fit option, it is possible to extrapolate values for sample intensities not falling within the limits of the standard curve. It is up to the researcher to decide the best method for calculating values for unknown samples using this assay. The theoretical limit of detection for each cytokine using the Mouse Th1/Th2/Th17 Cytokine Kit is defined as the corresponding concentration at two standard deviations above the median fluorescence of 30 replicates of the negative control (0 pg/mL).

Limit of detection data

Cytokine	Limit of detection (pg/mL)
IL-2	0.1
IL-4	0.03
IL-6	1.4
IFN-γ	0.5
TNF	0.9
IL-17A	0.8
IL-10	16.8

Recovery

About this topic This topic describes how a recovery experiment was performed and the results of the experiment.

How performed Individual cytokine protein was spiked into various matrices at three different levels within the assay range. The cell culture medium used in these experiments was not diluted before addition of the cytokine protein. The pooled mouse serum samples in these experiments were diluted 1:4 in Assay Diluent before addition of the cytokine protein. The spiked samples were assayed and the results were compared with the expected values.

Recovery data

Cytokine	Matrix	Average % Recovery	Range (%)
IL-2	Media Serum	83 81	81–87 79–85
IL-4	Media Serum	83 38	79–91 35–45
IL-6	Media Serum	83 79	83–84 77–83
IFN-γ	Media Serum	84 76	81–86 72–82
TNF	Media Serum	97 69	94–102 63–76
IL-17A	Media Serum	82 61	80–85 58–63
IL-10	Media Serum	97 78	78–121 74–80

Linearity

About this topic This topic describes how a linearity experiment was performed and the results of the experiment.

How performed In two experiments, the following matrices were spiked with IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A, and IL-10 and then were serially diluted with Assay Diluent.

Linearity data

Cytokine	Matrix	Sample dilution	Detected (pg/mL)	Average % of expected
IL-2	Media	1:2 1:4 1:8	1089.6 490.3 244.3	100 90 90
	Serum	1:2 1:4 1:8	1045.1 491.9 250.0	100 94 96
IL-4	Media	1:2 1:4 1:8	1124.2 496.5 251.1	100 88 89
	Serum	1:2 1:4 1:8	549.2 324.8 189.3	100 118 138
IL-6	Media	1:2 1:4 1:8	1008.5 496.1 245.4	100 98 97
	Serum	1:2 1:4 1:8	957.8 476.7 242.0	100 100 101

Cytokine	Matrix	Sample dilution	Detected (pg/mL)	Average % of expected
IFN-γ	Media	1:2 1:4 1:8	1017.5 503.7 263.3	100 99 104
	Serum	1:2 1:4 1:8	891.2 480.2 256.0	100 108 115
TNF	Media	1:2 1:4 1:8	1208.3 572.6 295.2	100 95 98
	Serum	1:2 1:4 1:8	777.9 417.3 222.4	100 107 114
IL-17A	Media	1:2 1:4 1:8	999.5 450.4 234.0	100 90 94
	Serum	1:2 1:4 1:8	735.1 418.0 230.6	100 114 125
IL-10	Media	1:2 1:4 1:8	959.5 490.5 294.0	100 102 123
	Serum	1:2 1:4 1:8	833.3 485.2 270.9	100 116 130

Specificity

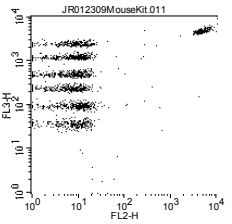
About this topic This topic describes how a specificity experiment was performed and the results of the experiment.

How performed The antibodies used in the Mouse Th1/Th2/Th17 Cytokine Kit have been screened for specific reactivity with their specific cytokines. Analysis of samples containing only a single recombinant cytokine protein

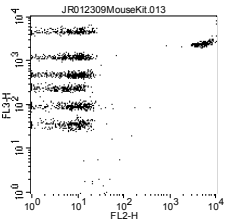
found no cross-reactivity or background detection of cytokine in other Capture Bead populations using this assay.

Specificity data

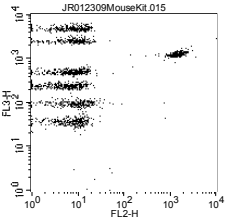
Sample data containing only a single recombinant cytokine protein was acquired using BD CellQuest software.



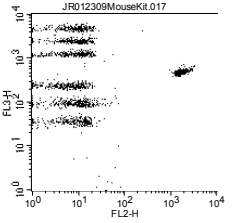
Mouse IL-2



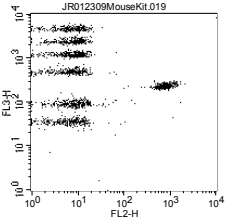
Mouse IL-4



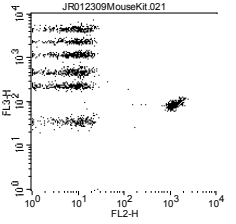
Mouse IL-6



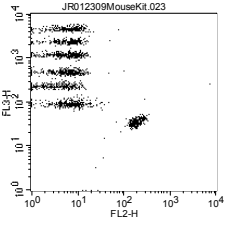
Mouse IFN-γ



Mouse TNF



Mouse IL-17A



Mouse IL-10

Precision

About this topic

This topic describes how intra-assay precision and inter-assay precision experiments were performed and the results of each experiment.

Intra-assay precision

Ten replicates of each of three different levels of IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A, and IL-10 were tested.

Cytokine	Sample	Mean (pg/mL)	Standard deviation	%CV
IL-2	Sample 1 Sample 2 Sample 3	71.1 292.0 1238.7	1.7 7.9 42.4	2 3 3
IL-4	Sample 1 Sample 2 Sample 3	70.6 291.8 1357.9	2.6 7.8 39.7	4 3 3
IL-6	Sample 1 Sample 2 Sample 3	79.1 315.0 1206.8	4.3 18.1 35.6	5 6 3
IFN-γ	Sample 1 Sample 2 Sample 3	79.6 313.7 1233.6	1.9 9.6 39.9	2 3 3
TNF	Sample 1 Sample 2 Sample 3	77.9 291.6 1172.2	3.7 10.7 45.4	5 4 4
IL-17A	Sample 1 Sample 2 Sample 3	82.8 316.6 1253.2	2.8 7.8 28.8	3 2 2
IL-10	Sample 1 Sample 2 Sample 3	89.5 307.5 1115.3	18.7 34.8 39.1	21 11 4

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Inter-assay precision

Three different levels of IL-2, IL-4, IL-6, IFN-γ, TNF, IL-17A, and IL-10 were tested in four experiments conducted by four different operators.

Cytokine	Sample	Mean (pg/mL)	Standard deviation	%CV
IL-2	Sample 1 Sample 2 Sample 3	76.0 299.2 1283.2	3.5 10.0 44.1	5 3 3
IL-4	Sample 1 Sample 2 Sample 3	76.2 299.1 1333.1	4.5 8.5 85.0	6 3 6
IL-6	Sample 1 Sample 2 Sample 3	81.4 314.7 1228.4	4.6 13.8 47.7	6 4 4
IFN-γ	Sample 1 Sample 2 Sample 3	81.1 313.7 1240.8	2.9 9.8 39.2	4 3 3
TNF	Sample 1 Sample 2 Sample 3	82.7 298.8 1224.5	5.8 13.3 65.4	7 4 5
IL-17A	Sample 1 Sample 2 Sample 3	84.4 316.1 1252.0	3.6 7.5 54.9	4 2 4
IL-10	Sample 1 Sample 2 Sample 3	94.8 342.6 1197.7	19.4 37.9 66.9	20 11 6

7

Reference

This section covers the following topics:

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Troubleshooting

About this topic This topic provides helpful information for specific problems that you might encounter while performing the Mouse Th1/Th2/Th17 Cytokine assay.

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

Problem	Recommended actions
Variation between duplicate samples	Vortex Capture Beads before pipetting. Beads can aggregate.
Low bead number in samples	Avoid aspiration of beads during wash step. Do not wash or resuspend beads in volumes higher than recommended volumes.
High background	Test various sample dilutions. The sample might be too concentrated. Remove excess Mouse Th1/Th2/TH17 PE Detection Reagent by increasing the number of wash steps, since background may be due to non-specific binding.
Little or no protein detected in sample	Sample may be too dilute. Try various sample dilutions.
Less than seven bead populations observed during analysis, or distribution is unequal	Ensure that equal volumes of beads were added to each assay tube. Vortex Capture Bead vials before taking aliquots. Once Capture Beads are mixed, vortex to ensure beads are distributed throughout solution.
Debris (FSC/SSC) during sample acquisition	Increase FSC threshold or further dilute samples. Increase number of wash steps, if necessary.

Problem	Recommended actions
Overlap of bead fluorescence (FL3) during acquisition	Samples might have very high cytokine concentration. Ensure instrument settings have been optimized using Cytometer Setup Beads.
Standards show low fluorescence or poor standard curve	Ensure that all components are properly prepared and stored. Use new vial of standard with each experiment and once reconstituted, do not use after 12 hours. Ensure that incubation times were of proper length.
All samples are positive or above high standard mean fluorescence value	Dilute samples further. Samples might be too concentrated.

References

About this topic This topic contains a list of the publications related to the information in this manual.

References

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2. Camilla C, Defoort JP, Delaage M, Auer R, Quintana J, Lary T, Hamelik R, Prato S, Casano B, Martin M, Fert V. A new flow cytometry-based multi-assay system. 1. Application to cytokine immunoassays. *Cytometry Suppl*. 1998;8:132.
3. Carson R, Vignali D. Simultaneous quantitation of fifteen cytokines using a multiplexed flow cytometric assay. *J Immunol Methods*. 1999;227:41–52.
4. Chen R, Lowe L, Wilson JD, et al. Simultaneous quantification of six human cytokines in a single sample using microparticle-based flow cytometric technology. *Clin Chem*. 1999;45:1693–1694.

5. Collins DP, Luebering BJ, Shaut DM. T-lymphocyte functionality assessed by analysis of cytokine receptor expression, intracellular cytokine expression, and femtomolar detection of cytokine secretion by quantitative flow cytometry. *Cytometry.* 1998;33:249–255.

6. Fulton RJ, McDade RL, Smith PL, Kienker LJ, Kettman JR Jr. Advanced multiplexed analysis with the FlowMetrix system. *Clin Chem.* 1997;43:1749–1756.

7. Kricka LJ. Simultaneous multianalyte immunoassays. In Immunoassay. Diamandis EP, Christopoulos TK, eds. Academic Press. 1996:389–404.

8. Lund-Johansen F, Davis K, Bishop J, de Waal Malefyt R. Flow cytometric analysis of immunoprecipitates: High-throughput analysis of protein phosphorylation and protein-protein interactions. *Cytometry.* 2000;39:250–259.

9. McHugh TM. Flow microsphere immunoassay for the quantitative and simultaneous detection of multiple soluble analytes. *Methods Cell Biol.* 1994;42:575–595.

10. Oliver KG, Kettman JR, Fulton RJ. Multiplexed analysis of human cytokines by use of the FlowMetrix system. *Clin Chem.* 1998;44:2057–2060.

11. Stall A, Sun Q, Varro R, Lowe L, Crowther E, Abrams B, Bishop J, Davis K. A single tube flow cytometric multibead assay for isotyping mouse monoclonal antibodies. Abstract LB77. Experimental Biology Meeting 1998 (late-breaking abstracts).

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Europe
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Asia/Pacific
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Latin America/Caribbean
0800.771.7157

BD Biosciences
2350 Qume Drive
San Jose, CA 95131-1807
Toll free: 877.232.8995 (US)
Tel: 408.432.9475
Fax: 408.954-2347
bdbiosciences.com

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