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

Instruction Manual

Catalog No. 560484

BD

History

Revision	Date	Change Made
647210	2/09	New document

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

For Research Use Only. Not for use in diagnostic or therapeutic procedures.

Contents

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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) Human Th1/Th2/Th17 Cytokine Kit (Catalog No. 560484), and provides background for understanding the kit's components and how they work.

Use of the kit

The BD CBA Human Th1/Th2/Th17 Cytokine Kit can be used to measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Tumor Necrosis Factor (TNF), Interferon-γ (IFN-γ), and Interleukin-17A (IL-17A) 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, EDTA plasma, 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 Human 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, IL-10, TNF, IFN-γ, and IL-17A 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.

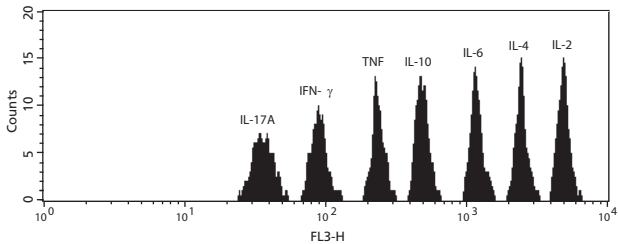

Figure 1

During the assay procedure, you will mix the cytokine capture beads with the 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 Human 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 42).

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.

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 Human Th1/Th2/Th17 Cytokine Kit.

Contents

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

Vial label	Reagent	Quantity
A1	Human IL-2 Capture Beads	1 vial, 0.8 mL
A2	Human IL-4 Capture Beads	1 vial, 0.8 mL
A3	Human IL-6 Capture Beads	1 vial, 0.8 mL

Vial label	Reagent	Quantity
A4	Human IL-10 Capture Beads	1 vial, 0.8 mL
A5	Human TNF Capture Beads	1 vial, 0.8 mL
A6	Human IFN-γ Capture Beads	1 vial, 0.8 mL
A7	Human IL-17A Capture Beads	1 vial, 0.8 mL
B	Human Th1/Th2/Th17 PE Detection Reagent	1 vial, 4 mL
C	Human 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
H	Serum Enhancement Buffer	1 bottle, 10 mL

Bead reagents

Human 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

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

Human Th1/Th2/Th17 Cytokine Standards (C): Two vials containing lyophilized recombinant human 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 Human Th1/Th2/Th17 Cytokine Standards and to dilute unknown samples.

Serum Enhancement Buffer (H): A single 10-mL bottle of a buffered protein solution (1x) used to dilute mixed Capture Beads when testing serum or plasma 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, G, and H 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 Human Th1/Th2/Th17 Cytokine Standards (page 14)
2	Mixing Human 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 Human 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 mixed capture beads (when analyzing serum or plasma samples only)	30 minutes
Preparing Cytometer Setup Beads	30 minutes
Staining samples for analysis	3 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 Human 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 Human Th1/Th2/Th17 Cytokine Standards

About this topic

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

Purpose of this procedure

The Human 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 prepare fresh cytokine standards to run with each experiment. Do not store or reuse reconstituted or diluted standards.

Procedure

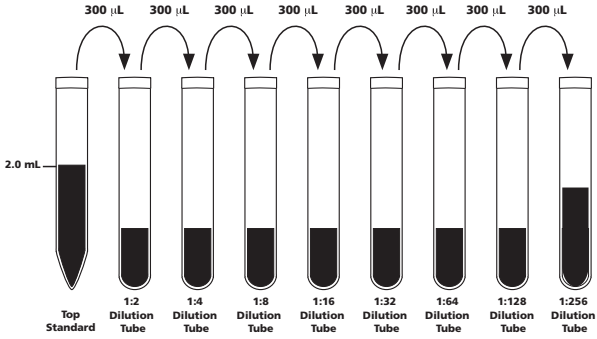
To reconstitute and serially dilute the standards:

1. Open one vial of lyophilized Human 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 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 Human 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 Human Th1/Th2/Th17 Cytokine Capture Beads (page 16).

Related topics

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

Mixing Human 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.

Mixing the beads

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.

Resuspending the beads

If you are using serum or plasma samples, you must perform this procedure. This procedure is optional for all other sample types.

To resuspend the Capture Beads in Serum Enhancement Buffer:

1. Centrifuge the mixed Capture Beads at 200*g* for 5 minutes.

2. Carefully aspirate and discard the supernatant.
3. Resuspend the mixed Capture Beads pellet in Serum Enhancement Buffer (equal to the volume removed in step 2) and vortex thoroughly.
4. Incubate the mixed Capture Beads for 30 minutes at room temperature, protected from light.

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 Human 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/plasma 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.

   Note: Optimal recovery from serum samples typically requires a 1:4 dilution.

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 Human Th1/Th2/Th17 Cytokine samples (page 28), and you can perform instrument setup during the 3-hour staining incubation.

4

Cytometer setup

This section covers the following topics:

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

Actions 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 Human Th1/Th2/Th17 Cytokine Kit when using the dual-laser protocol.

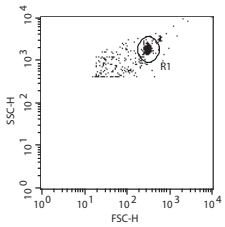
Running the Cytometer Setup Beads

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.

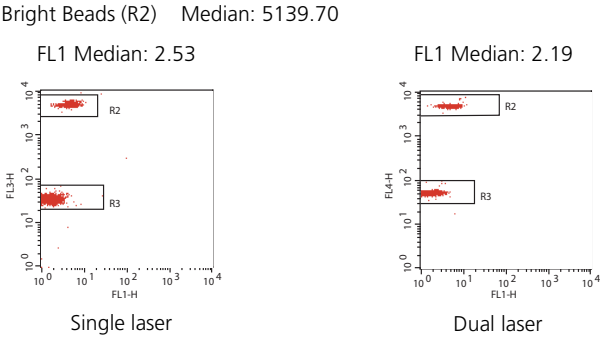


3. Make the following adjustments:

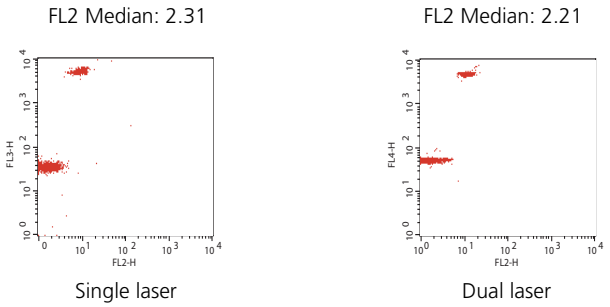
   Adjust the FL3 (or FL4 for dual-laser cytometer) PMT voltage so that the median of the top FL3 (FL4 for dual laser) bead population's intensity is approximately 5000.

   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.



4. Adjust the FL2 PMT voltage so that the median of FL2 is approximately 2.0 to 2.5.



5. Depending on your cytometer:

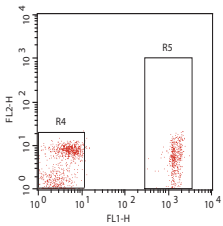
   - Dual-laser cytometer users save and print optimized instrument settings. Proceed to Staining Human Th1/Th2/Th17 Cytokine samples (page 28).
   - Single-laser cytometer users continue with Adjusting compensation (page 25).

Adjusting compensation

To adjust compensation if using a single-laser cytometer:

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.

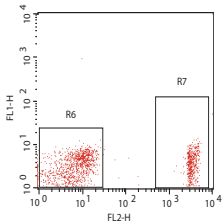
   Adjust the FL2–%FL1 compensation setting so that the median of R5 is equal to the median of R4.



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.

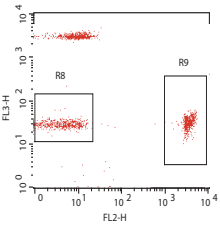
   Adjust the FL1–%FL2 compensation setting so that the median of R7 is equal to the median of R6.



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 Human Th1/Th2/Th17 Cytokine samples (page 28).

5

Assay procedure

This section covers the following topics:

Staining Human 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 Human Th1/Th2/Th17 Cytokine Standards (page 14).
- Mix the Capture Beads as described in Mixing Human Th1/Th2/Th17 Cytokine Capture Beads (page 16). Be sure to follow the appropriate procedure (cell culture supernatant vs serum/plasma) for your sample type.
- 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 Human 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

Tube label	Concentration (pg/mL)	Cytokine Standard dilution
7	625	1:8
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 tubes.
4. Add 50 µL of the Human Th1/Th2/Th17 PE Detection Reagent to all assay tubes.
5. Incubate the assay tubes for 3 hours at room temperature, protected from light.

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

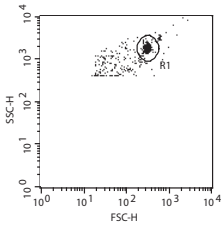
Procedure

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.

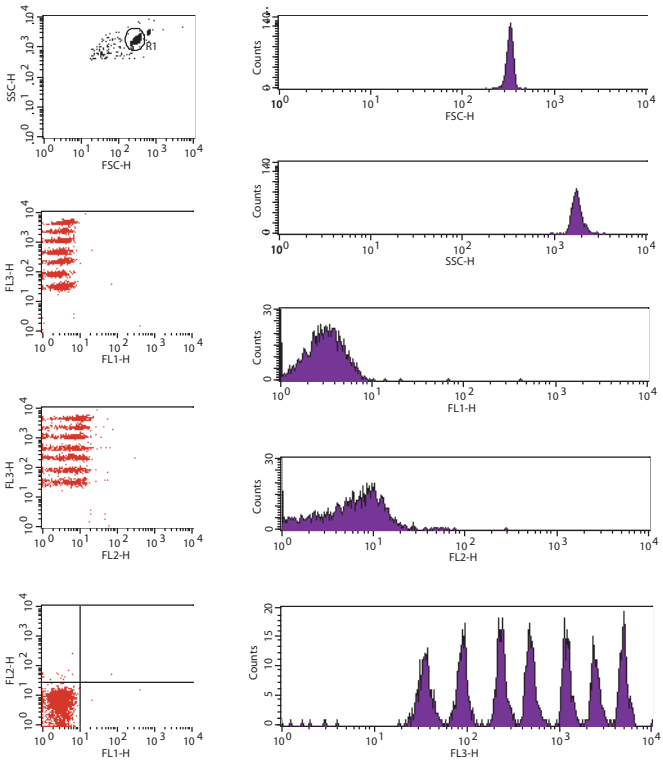
6. If you are running a dual-laser instrument, click the Parameters Saved button. Ensure that only FSC-H, SSC-H, FL1-H, FL2-H, and FL4-H are selected. It is important that additional detectors be turned off. Click OK.

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).

Data Analysis

About this topic

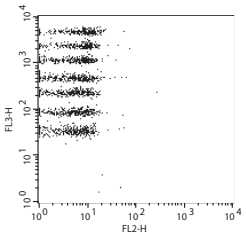
This topic shows examples of Human Th1/Th2/Th17 Cytokine data acquired using BD CellQuest software, and specifies the software required to analyze the data.

How to analyze

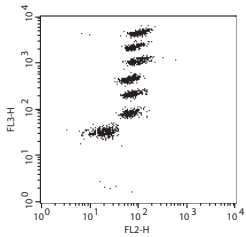
Analyze Human Th1/Th2/Th17 Cytokine data using FCAP Array software. For instructions on using FCAP Array software, go to bdbiosciences.com/docs/FCAP_Array_analysis_of_CBA_Kits.pdf.

Typical data

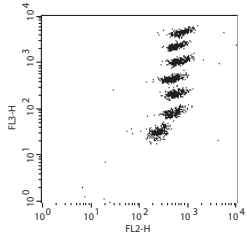
The following data, acquired using BD CellQuest software, shows standards and detectors alone.



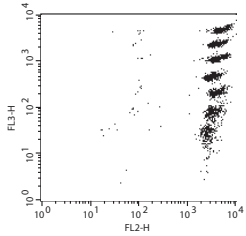
Negative control (0 pg/mL)



Standard 80 pg/mL



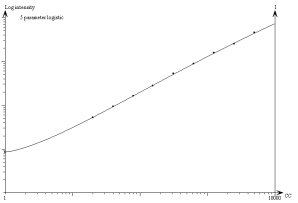
Standard 625 pg/mL



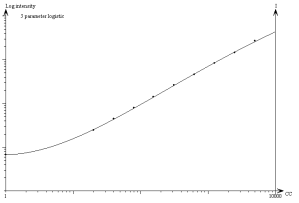
Standard 5000 pg/mL

Standard curve examples

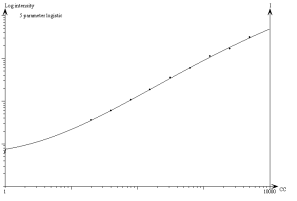
The following graphs represent standard curves from the Human Th1/Th2/Th17 Cytokine Standards.



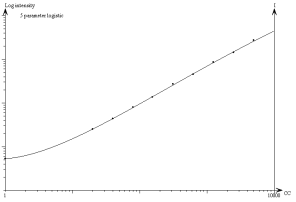
IL-2



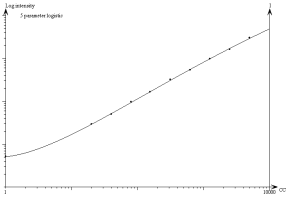
IL-4



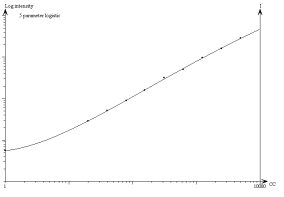
IL-6



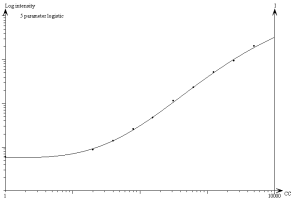
IL-10



TNF



IFN-γ



IL-17A

6

Performance

This section covers the following topics:

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 Human 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 Human 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	2.6
IL-4	4.9
IL-6	2.4
IL-10	4.5
TNF	3.8
IFN-γ	3.7
IL-17A	18.9

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 spiked samples were assayed and the results were compared with the expected values. The cell culture medium used in these experiments was not diluted before addition of the cytokine protein. Pooled human serum and pooled human plasma samples were diluted 1:4 in Assay Diluent before addition of cytokine protein. The plasma samples in these experiments were EDTA treated.

Recovery data

Cytokine	Matrix	Average % Recovery	Range (%)
IL-2	Media Serum Plasma	83 86 84	72–95 83–91 78–92
IL-4	Media Serum Plasma	81 91 87	75–87 87–94 85–88
IL-6	Media Serum Plasma	86 90 93	79–92 88–92 91–98
IL-10	Media Serum Plasma	86 95 91	80–92 93–96 89–94
TNF	Media Serum Plasma	88 95 95	81–95 93–97 92–98
IFN-γ	Media Serum Plasma	80 84 76	72–89 82–87 76–77
IL-17A	Media Serum Plasma	84 74 73	74–93 60–92 55–93

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, IL-10, TNF, IFN-γ, and IL-17A 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	1020.8 469.4 208.2	100 92 82
	Serum	1:2 1:4 1:8	1161.5 514.0 241.0	100 89 83
	Plasma	1:2 1:4 1:8	1001.4 480.2 232.9	100 96 93
IL-4	Media	1:2 1:4 1:8	958.4 464.2 222.0	100 97 93
	Serum	1:2 1:4 1:8	1119.9 523.8 263.7	100 94 94
	Plasma	1:2 1:4 1:8	937.5 494.6 250.3	100 106 107
IL-6	Media	1:2 1:4 1:8	1101.5 498.1 236.7	100 90 86
	Serum	1:2 1:4 1:8	1203.6 567.8 275.7	100 94 92
	Plasma	1:2 1:4 1:8	1063.0 577.2 272.5	100 109 103

Cytokine	Matrix	Sample dilution	Detected (pg/mL)	Average % of expected
IL-10	Media	1:2 1:4 1:8	1049.2 506.0 240.9	100 96 92
	Serum	1:2 1:4 1:8	1167.6 543.3 270.1	100 93 93
	Plasma	1:2 1:4 1:8	1013.6 506.8 250.1	100 100 99
TNF	Media	1:2 1:4 1:8	1077.0 518.4 238.5	100 96 89
	Serum	1:2 1:4 1:8	1277.8 574.2 273.2	100 90 86
	Plasma	1:2 1:4 1:8	1084.1 573.3 273.5	100 106 101
IFN-γ	Media	1:2 1:4 1:8	946.1 441.6 219.7	100 93 93
	Serum	1:2 1:4 1:8	1039.1 466.9 219.0	100 90 84
	Plasma	1:2 1:4 1:8	857.7 451.3 214.6	100 105 100

Cytokine	Matrix	Sample dilution	Detected (pg/mL)	Average % of expected
IL-17A	Media	1:2 1:4 1:8	1106.7 521.8 244.8	100 94 88
	Serum	1:2 1:4 1:8	937.3 496.4 256.8	100 106 110
	Plasma	1:2 1:4 1:8	752.9 466.0 226.4	100 124 120

Specificity

About this topic

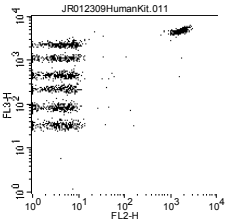
This topic describes how a specificity experiment was performed and the results of the experiment.

How performed

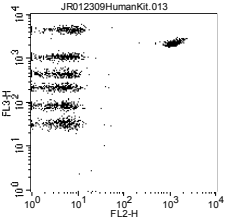
The antibodies used in the Human Th1/Th2/Th17 Cytokine Kit have been screened for specific reactivity with their specific cytokines. Analysis of samples containing only a single recombinant cytokine protein found no cross-reactivity or background detection of cytokine in other Capture Bead populations using this assay.

Specificity data

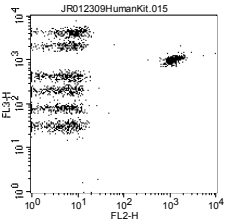
Sample data containing only a single recombinant cytokine protein was acquired using BD CellQuest software.



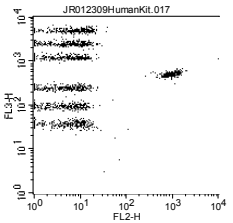
Human IL-2



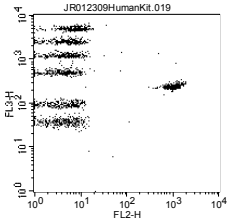
Human IL-4



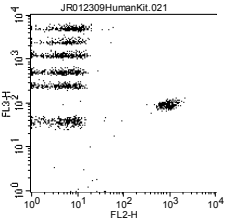
Human IL-6



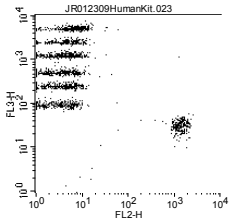
Human IL-10



Human TNF



Human IFN-γ



Human IL-17A

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, IL-10, TNF, IFN-γ, and IL-17A were tested.

Cytokine	Sample	Mean (pg/mL)	Standard deviation	%CV
IL-2	Sample 1 Sample 2 Sample 3	67.8 279.1 1238.5	2.9 14.8 46.0	4 5 4
IL-4	Sample 1 Sample 2 Sample 3	78.1 294.5 1231.2	3.8 10.2 25.8	5 3 2
IL-6	Sample 1 Sample 2 Sample 3	75.8 303.1 1284.1	4.5 14.1 55.5	6 5 4
IL-10	Sample 1 Sample 2 Sample 3	76.2 296.3 1272.2	4.1 11.3 46.8	5 4 4
TNF	Sample 1 Sample 2 Sample 3	75.1 302.6 1316.9	6.0 20.4 74.5	8 7 6
IFN-γ	Sample 1 Sample 2 Sample 3	69.6 280.9 1233.7	2.4 11.4 53.1	3 4 4
IL-17A	Sample 1 Sample 2 Sample 3	76.9 305.7 1308.9	3.6 7.8 52.8	5 3 4

Inter-assay precision

Three different levels of IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A 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	71.0 290.0 1230.3	6.3 20.3 82.0	9 7 7
IL-4	Sample 1 Sample 2 Sample 3	76.9 297.7 1217.6	8.1 18.6 57.6	11 6 5
IL-6	Sample 1 Sample 2 Sample 3	77.7 297.8 1254.4	10.4 25.3 89.0	13 8 7
IL-10	Sample 1 Sample 2 Sample 3	77.8 296.0 1245.8	8.3 18.8 93.7	11 6 8
TNF	Sample 1 Sample 2 Sample 3	77.8 300.0 1263.7	9.4 26.9 96.8	12 9 8
IFN-γ	Sample 1 Sample 2 Sample 3	74.4 291.2 1239.8	8.3 26.6 95.1	11 9 8
IL-17A	Sample 1 Sample 2 Sample 3	75.1 303.7 1272.8	9.4 22.7 76.8	12 7 6

7

Reference

This section covers the following topics:

Troubleshooting

About this topic This topic provides helpful information for specific problems that you might encounter while performing the Human 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 Human Th1/Th2/TH17 II 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.
Overlap of bead fluorescence (FL3) during acquisition	Samples might have very high cytokine concentration. Ensure instrument settings have been optimized using Cytometer Setup Beads.

Problem	Recommended actions
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.
Biohazardous samples	You may treat samples briefly with 1% paraformaldehyde before acquiring samples on the flow cytometer. However, this may affect assay performance and should be validated by the user.

References

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

References

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

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13. Dotti G, Salvodo B, Takahashi S, et al. Adenovector-induced expression of human-CD40-ligand (hCD40L) by multiple myeloma cells: A model for immunotherapy. *Exp Hematol*. 2001;29:952–961.

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