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

Instruction Manual

Catalog No. 551287

BD

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Regulatory information

BD flow cytometers are class 1 laser products.

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

History

Revision	Date	Change made
23-12494-00 Rev. 01	11/2010	New document
23-12494-01 Rev. 01	6/2011	Added serum protein statement

Contents

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About this kit

This section covers the following topics:

Purpose of the kit

Use of the kit

The BD™ CBA Mouse Th1/Th2 Cyokine Kit (Catalog No. 551287) can be used to measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interferon-γ (IFN-γ), and Tumor Necrosis Factor (TNF) 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 80 samples.

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.

Principles of this assay

The BD CBA Mouse Th1/Th2 Cytokine Kit uses bead array technology to simultaneously detect multiple cytokine proteins in research samples. Five bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-2, IL-4, IL-5, IFN-γ, and TNF proteins. The five bead populations are mixed together to form the bead array, which is resolved in a red channel of a flow cytometer.

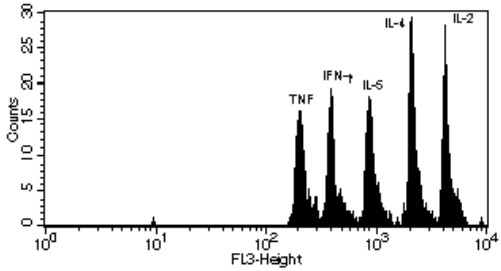

Figure 1

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 fluorescence 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-fifty the quantity necessary for conventional ELISA assays due to the detection of five 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 five conventional ELISAs.

Limitations

Assay limitations

The theoretical limit of detection of the BD CBA Mouse Th1/Th2 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 slightly. See Theoretical limit of detection (page 32) and Precision (page 37).

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

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.

Kit contents

Contents

The BD CBA Mouse Th1/Th2 Cytokine Kit contains the following components sufficient for 80 tests.

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-5 Capture Beads	1 vial, 0.8 mL
A4	Mouse IFN-γ Capture Beads	1 vial, 0.8 mL
A5	Mouse TNF Capture Beads	1 vial, 0.8 mL
B	Mouse Th1/Th2 PE Detection Reagent	1 vial, 4 mL
C	Mouse Th1/Th2 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

Vial label	Reagent	Quantity
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–A5): An 80-test vial of each specific capture bead (A1–A5). The capture beads, having discrete fluorescence intensity characteristics, are distributed from brightest (A1) to dimmest (A5).

Cytometer Setup Beads (D): A 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 PE Detection Reagent (B): An 80-test vial of PE-conjugated anti-mouse IL-2, IL-4, IL-5, IFN-γ, and TNF antibodies that is formulated for use at 50 µL/test.

Mouse Th1/Th2 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

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

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

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

Storage and handling

Storage

Store all kit components at 2 to 8°C. Do not freeze.

Warning

Components A1-A5, B, D, E1-E2, F, and G contain sodium azide. Sodium azide yields a highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

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Before you begin

This section covers the following topics:

Workflow overview

Workflow

The overall workflow consists of the following steps.

Step	Description
1	Preparing Mouse Th1/Th2 Cytokine Standards (page 18)
2	Mixing Mouse Th1/Th2 Cytokine Capture Beads (page 20)
3	Diluting samples (page 21)
4	Performing instrument setup with Cytometer Setup Beads (instructions can be found at bdbiosciences.com/cbasetup) **Note:** Can be performed during the incubation in step 5.
5	Performing the Mouse Th1/Th2 Cytokine Assay (page 24)
6	Acquiring samples (instructions can be found at bdbiosciences.com/cbasetup)
7	Data analysis (page 28)

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
Performing the assay	2 hours

Required materials

Materials required but not provided

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

- A dual-laser flow cytometer equipped with a 488-nm or 532-nm and a 633-nm or 635-nm laser capable of distinguishing 576-nm, 660-nm, and >680-nm fluorescence. The following table lists examples of compatible instrument platforms.

Flow cytometer	Reporter channel	Bead channel
BD FACSArray™	Yellow	Red
BD FACSCanto™ platform BD™ LSR platform BD FACSAria™ platform	PE	APC
BD FACSCalibur™ (single laser) BD FACSCalibur (dual laser)	FL2	FL3 FL4
BD FACSVerse™	PE	CBA Red
Note: Visit bdbiosciences.com/cbasetup for setup protocols.		

- BD Falcon™ 12 × 75-mm sample acquisition tubes for a flow cytometer (Catalog No. 352008).
- 15-mL conical, polypropylene tubes (BD Falcon, Catalog No. 352097), or equivalent
- FCAP Array software (Catalog No. 652099 [PC]) or 645447 [Mac®]).

Materials required for plate loader-equipped flow cytometers

- Millipore MultiScreen$_{HTS}$-BV 1.2-µm Clear non-sterile filter plates [Catalog No. MSBVN1210 (10 pack) or MSBVN1250 (50 pack)]
- Millipore MultiScreen$_{HTS}$ Vacuum Manifold, (Catalog No. MSVMHTS00)
- MTS 2/4 Digital Stirrer, IKA Works, VWR (Catalog No. 82006-096)
- Vacuum source
- Vacuum gauge and regulator (if not using the recommended manifold)

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Assay preparation

This section covers the following topics:

Preparing Mouse Th1/Th2 Cytokine Standards

Purpose of this procedure

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

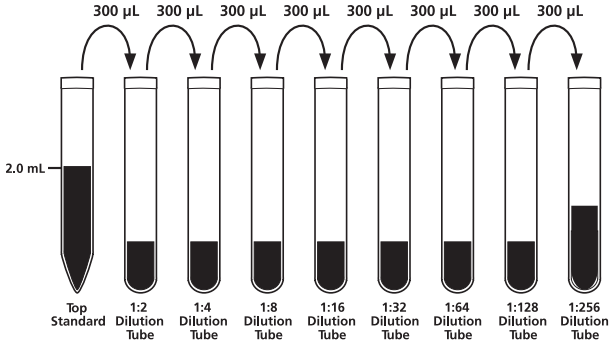
You must prepare fresh cytokine standards to run with each experiment. Do not store or reuse reconstituted or diluted standards.

Procedure

To reconstitute and serially dilute the standards:

1. Open one vial of lyophilized Mouse Th1/Th2 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 reconstituted protein by pipet only. Do not vortex or mix vigorously.
3. Label eight 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 into each of the tubes.
5. Perform a serial dilution:
    a. Transfer 300 µL from the Top Standard to the 1:2 dilution tube and mix thoroughly by pipet 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.



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

Concentration of standards

See the table in Performing the Mouse Th1/Th2 Cytokine Assay (page 24) for a listing of the concentrations (pg/mL) of all five recombinant proteins in each standard dilution.

Next step

Proceed to Mixing Mouse Th1/Th2 Cytokine Capture Beads (page 20).

Mixing Mouse Th1/Th2 Cytokine Capture Beads

Purpose of this procedure

The Capture Beads are bottled individually and it is necessary to pool the bead reagents (A1–A5) immediately before mixing them together with the standards, samples, and PE Detection reagent.

Procedure

To mix the Capture Beads:

1. Determine the number of assay tubes (including standards and controls) that are required for the experiment (for example, 8 unknowns, 9 cytokine standard dilutions, and 1 negative control = 18 assay tubes).

   Note: Extra tests of Capture Beads should be mixed to ensure that the necessary number of tests will be recovered from the mixed Capture Beads tube. Add an additional 2 to 3 assay tubes to the number determined in step 1 above before calculating the amount to add to the mixed Capture Beads tube in step 3.

2. Vigorously vortex each Capture Bead suspension for a few seconds before mixing.

   Note: The antibody-conjugated beads will settle out of suspension over time. Vortex the vial immediately 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" (for example, 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 Performing the Mouse Th1/Th2 Cytokine Assay (page 24). If you need to dilute samples having a high cytokine concentration, proceed to Diluting samples (page 21).

Diluting samples

Purpose of this procedure

The standard curve for each cytokine covers a defined set of concentrations from 20 to 5,000 pg/mL. It might be necessary to dilute test samples to ensure that their median 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 known high-cytokine concentration:

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

Next step

Perform instrument setup using the Cytometer Setup Beads. For details, go to bdbiosciences.com/cbasetup and select the appropriate flow cytometer under CBA Kits: Instrument Setup.

Or, if you wish to begin staining your samples for the assay, proceed to Performing the Mouse Th1/Th2 Cytokine Assay (page 24), and you can perform instrument setup during the 2-hour staining incubation.

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Assay procedure

This section covers the following topics:

Performing the Mouse Th1/Th2 Cytokine Assay

Before you begin

- Prepare the standards as described in Preparing Mouse Th1/Th2 Cytokine Standards (page 18).
- Mix the Capture Beads as described in Mixing Mouse Th1/Th2 Cytokine Capture Beads (page 20).
- If necessary, dilute the unknown samples. See Diluting samples (page 21).

Procedure for tubes

To perform the assay:

1. Vortex the mixed Capture Beads and add 50 µL to all assay tubes.
2. Add 50 µL of the Mouse Th1/Th2 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
8	1,250	1:4
9	2,500	1:2
10	5,000	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 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.
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.

Procedure for filter plates

To perform the assay:

1. Wet the plate by adding 100 µL of Wash Buffer to each well.
2. Place the plate on the vacuum manifold.
3. Aspirate for 2 to 10 seconds until the wells are drained.
4. Remove the plate from the manifold, then blot the bottom of the plate on paper towels.
5. Add 50 µL of each of the following to the wells in the filter plate:
   - Capture Beads (vortex before adding)
   - Standard or sample (add standards from the lowest concentration to the highest, followed by samples)
   - Mouse Th1/Th2 PE Detection Reagent
6. Cover the plate and shake it for 5 minutes at 1,100 rpm on a plate shaker.

7. Incubate the plate for 2 hours at room temperature on a non-absorbent, dry surface.

   Note: Place the plate on a non-absorbent, dry surface during incubation. Absorbent or wet surfaces can cause the contents of the wells to leak.

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

8. Remove the cover from the plate and apply the plate to the vacuum manifold.
9. Vacuum aspirate for 2 to 10 seconds until the wells are drained.
10. Remove the plate from the manifold, then blot the bottom of the plate on paper towels after aspiration.
11. Add 120 µL of Wash Buffer to each well to resuspend the beads.
12. Cover the plate and shake it for 2 minutes at 1,100 rpm before you begin sample acquisition.

Next step

Acquire the samples on the flow cytometer. For details, go to bdbiosciences.com/cbasetup and select the appropriate flow cytometer under CBA Kits: Instrument Setup.

CBA samples must be acquired on the same day they are prepared. Prolonged storage of samples, once the assay is complete, can lead to increased background and reduced sensitivity.

To facilitate the analysis of samples using the FCAP Array software, we recommend the following guidelines:

- Acquire standards from lowest (0 pg/mL) to highest (Top Standard) concentration, followed by the test samples.
- If running sample dilutions, acquire sequentially starting with the most concentrated sample.
- Store all FCS files (standards and samples) in a single folder.

When you are finished acquiring samples, proceed to Data analysis (page 28).

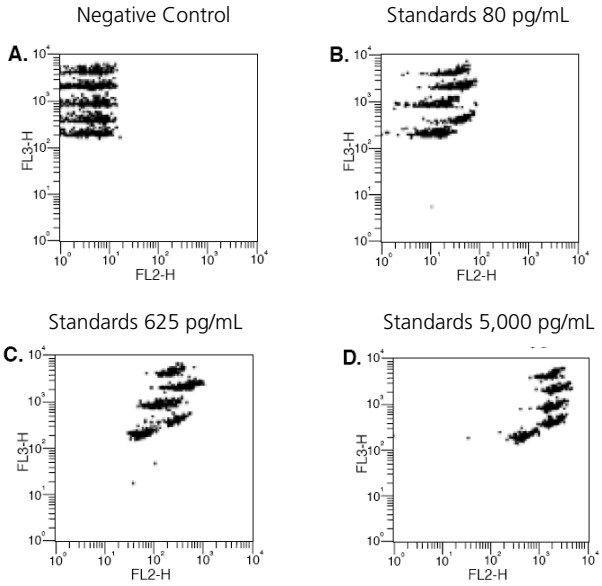
Data analysis

How to analyze

Analyze Mouse Th1/Th2 Cytokine data using FCAP Array software. For instructions on analysis, go to bdbiosciences.com/cbasetup and see the *FCAP Array Software User's Guide*.

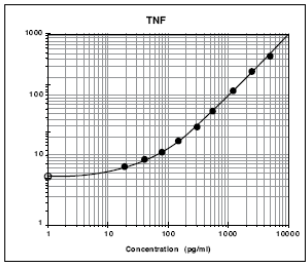
Typical data

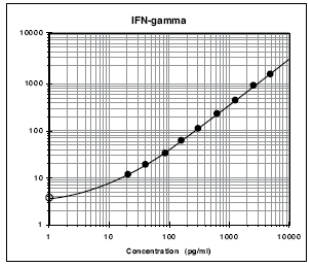
The following data, acquired using BD CellQuest™ software, shows standards and detectors alone.

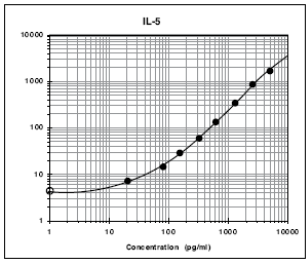


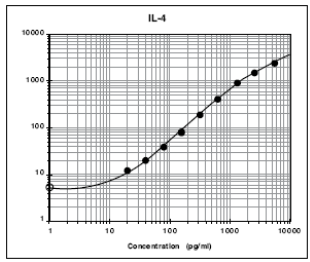
Standard curve examples

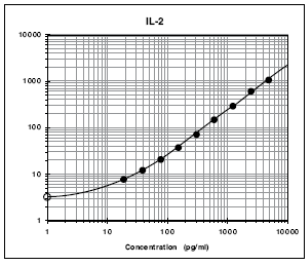
The following graphs represent standard curves from the BD CBA Mouse Th1/Th2 Cytokine Standards.











5

Performance

This section covers the following topics:

Theoretical limit of detection

Experiment details

The individual standard curve range for a given cytokine defines the minimum and maximum quantifiable levels (20 pg/mL and 5,000 pg/mL) using the BD CBA Mouse Th1/Th2 Cytokine Kit. 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 BD CBA Mouse Th1/Th2 Cytokine Kit is defined as the corresponding concentration at two standard deviations above the median fluorescence of 20 replicates of the negative control (0 pg/mL).

Limit of detection

Cytokine	Median Fluorescence	Standard deviation	Limit of detection (pg/mL)
IL-2	5.0	0.6	5.0
IL-4	4.8	0.6	5.0
IL-5	4.4	0.7	5.0
IFN-γ	4.5	0.6	2.5
TNF	4.5	0.6	6.3

Recovery

Experiment details

Individual cytokine protein was spiked into various matrices at three different levels within the assay range. The cell culture media 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 or 1:10 in Assay Diluent before addition of the cytokine protein. Results are compared with the same concentrations of the cytokines spiked in the Standard Diluent.

Recovery data

Cytokine	Matrix	Standard spike concentration (pg/mL)	Observed in given matrix (pg/mL)	% Recovery
IL-2	Pooled mouse sera 1:4 dilution	2,500 625 80	1,936.7 455.4 54.5	77% 73% 68%
	Pooled mouse sera 1:10 dilution	2,500 625 80	1,991.1 460 52.7	80% 74% 66%
	Cell culture media	2,500 625 80	2,303.4 592.9 67.7	92% 95% 85%
IL-4	Pooled mouse sera 1:4 dilution	2,500 625 80	1,002.5 142.7 18.6	40% 23% 23%
	Pooled mouse sera 1:10 dilution	2,500 625 80	1,724.3 243 27.7	69% 39% 35%
	Cell culture media	2,500 625 80	2,566.6 631.5 62.7	103% 101% 78%

Cytokine	Matrix	Standard spike concentration (pg/mL)	Observed in given matrix (pg/mL)	% Recovery
IL-5	Pooled mouse sera 1:4 dilution	2,500 625 80	2,328.1 532.6 58	93% 85% 72%
	Pooled mouse sera 1:10 dilution	2,500 625 80	2,235.7 505 51.7	89% 81% 65%
	Cell culture media	2,500 625 80	2,414.8 592.4 61.8	97% 95% 77%
IFN-γ	Pooled mouse sera 1:4 dilution	2,500 625 80	2,127.7 475.1 74.1	85% 76% 93%
	Pooled mouse sera 1:10 dilution	2,500 625 80	2,164.6 546 71.8	87% 87% 90%
	Cell culture media	2,500 625 80	2,328.3 612.7 74.4	93% 98% 93%
TNF	Pooled mouse sera 1:4 dilution	2,500 625 80	960.3 253.6 30.7	38% 41% 38%
	Pooled mouse sera 1:10 dilution	2,500 625 80	1,082.7 273 34.3	43% 44% 43%
	Cell culture media	2,500 625 80	2,244.1 572.1 64.6	90% 92% 81%

Linearity

Experiment details

In two experiments, the following matrices were spiked with IL-2, IL-4, IL-5, IFN-γ, and TNF and then were serially diluted with Assay Diluent.

Linearity data

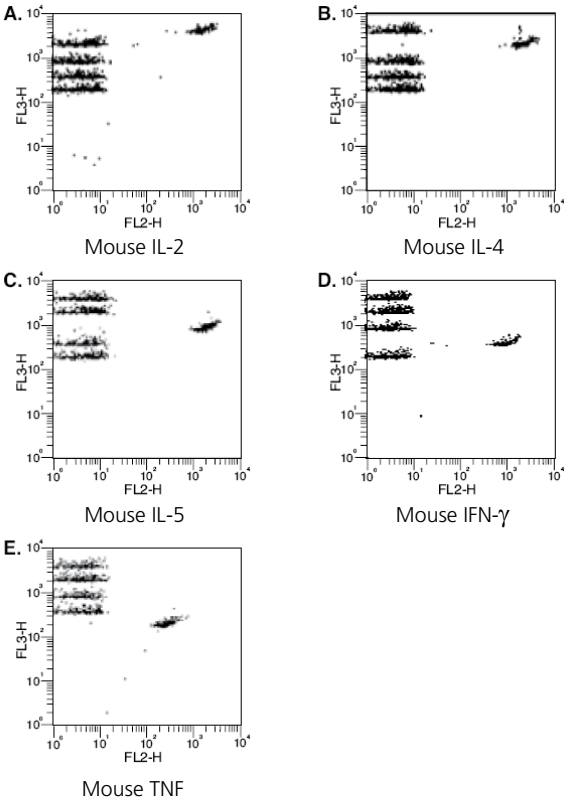
Matrix	Dilution	Observed (pg/mL)				
		IL-2	IL-4	IL-5	IFN-γ	TNF
Pooled mouse sera	1:4	3,422	2,421	3,946	4,108	2,019
	1:8	2,006	1,799	2,497	2,316	1,131
	1:16	1,021	1,052	1,167	1,159	600
	1:32	498	506	507	588	341
	1:64	222	232	197	289	192
	1:128	105	96	85	131	95
	1:256	52	42	34	66	50
	1:512	25	17	14	30	26
	1:1024	8.1	6	2	11	6
	Slope	1.07	1.10	1.31	1.05	0.97
Cell culture media	Neat	4,202	2,797	4,186	4,679	4,862
	1:2	2,696	2,551	2,765	2,598	2,741
	1:4	1,328	1,705	1,354	1,326	1,311
	1:8	632	814	610	634	576
	1:16	298	350	258	311	295
	1:32	135	147	117	148	146
	1:64	63	56	46	74	64
	1:128	26	23	18	33	21
	1:256	13	11	8	16	10
	Slope	1.07	1.08	1.17	1.03	1.12

Specificity

Experiment details

The antibodies used in the BD CBA Mouse Th1/Th2 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



Mouse IL-2

Mouse IL-4

Mouse IL-5

Mouse IFN-γ

Mouse TNF

Precision

Intra-assay precision

Ten replicates of each of three different levels of IL-2, IL-4, IL-5, IFN-γ, and TNF were tested.

Cytokine	Actual Mean Conc (pg/mL)	Standard deviation	%CV
IL-2	58 469 2,219	3 26 62	6% 6% 3%
IL-4	59 557 2,648	2 37 73	4% 7% 3%
IL-5	50 463 2,493	4 26 83	9% 6% 3%
IFN-γ	73 594 2,565	2 16 108	3% 3% 4%
TNF	59 482 2,138	7 30 57	11% 6% 3%

Inter-assay precision

Three different levels of IL-2, IL-4, IL-5, IFN-γ, and TNF (80, 625, and 2,500 pg/mL) were tested in four experiments conducted by four different operators.

Note: The number of replicates refers to the total number of assay tubes tested at a given concentration of protein.

Cytokine	Number of replicates	Actual Mean Conc (pg/mL)	Standard deviation	%CV
IL-2	8	63	4.9	8%
	8	544	44	8%
	8	2,362	218	9%
IL-4	8	61	4	7%
	8	565	58	10%
	8	2,634	283	11%
IL-5	8	54	7	14%
	8	525	56	11%
	8	2,376	294	12%
IFN-γ	8	77	6	8%
	8	602	44	7%
	8	2,419	194	8%
TNF	8	73	13	18%
	8	551	43	8%
	8	2,341	198	8%

6

Reference

This section covers the following topics:

Troubleshooting

Recommended actions

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

Note: For best performance, vortex samples immediately before analyzing on a flow cytometer.

Problem	Suggested solution
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 may be too concentrated. Remove excess Mouse Th1/Th2 PE Detection Reagent by increasing the number of wash steps since the background may be due to non-specific binding.
Little or no detection of protein in sample.	Sample may be too dilute. Try various sample dilutions.
Less than six bead populations are 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 that the beads are distributed evenly throughout the solution.
Debris (FSC/SSC) during sample acquisition. Also for plasma samples.	Increase the FSC threshold or further dilute samples. Increase the number of wash steps if necessary. Make a tighter FSC/SSC region gate around the bead population.
Overlap of bead population fluorescence (FL3) during acquisition.	This may occur in samples with very high cytokine concentration. Ensure that instrument settings have been optimized using the Cytometer Setup Beads.

Problem	Suggested solution
Standards assay tubes show low fluorescence or poor standard curve.	Check that all components are properly prepared and stored. Use a 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 the high standard mean fluorescence value.	Dilute the samples further. The samples may be too concentrated.
Biohazardous samples.	It is possible to treat samples briefly with 1% paraformaldehyde before analyzing on the flow cytometer. However, this may affect assay performance and should be validated by the user.

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23-12494-01 Rev. 01