BD™ Cytometric Bead Array (CBA) Non-Human Primate Th1/Th2 Cytokine Kit

Instruction Manual

Catalog No. 557800

BD

Trademarks

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Mac is a trademark of Apple Computer, Inc., registered in the US and other countries.

Falcon® is a registered trademark of Corning Incorporated.

History

Revision	Date	Change made
23-12718-00 Rev. 01	1/2011	Initial release
23-12718-01 Rev. 01	2/2012	Updated image
23-12718-02	2/2013	Updated patent information
Rev. 2	4/2015	Update hazard statements

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Contents

Chapter 1: About this kit 5
Purpose of this kit 6
Limitations 8
Kit contents 9
Storage and handling 11
Chapter 2: Before you begin 13
Workflow overview 14
Required materials 15
Chapter 3: Assay preparation 17
Preparing Human Th1/Th2 Cytokine Standards 18
Mixing NHP Th1/Th2 Cytokine Capture Beads 20
Diluting samples 21
Chapter 4: Assay procedure 23
Performing the Non-Human Primate Th1/Th2 Cytokine Assay 24
Data analysis 27
Chapter 5: Performance 29
Theoretical limit of detection 30
Recovery 31
Linearity 37
Specificity 43
Precision 44
Chapter 6: Reference 47
Troubleshooting 48
References 50

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1

About this kit

This section covers the following topics:

- Purpose of this kit (page 6)
- Limitations (page 8)
- Kit contents (page 9)
- Storage and handling (page 11)

Purpose of this kit

Uses of the kit

The BD™ CBA Non-Human Primate (NHP) Th1/Th2 Cytokine Kit (Catalog No. 557800) can be used to quantitatively measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-6 (IL-6), Tumor Necrosis Factor (TNF), and Interferon-γ (IFN-γ) protein levels in a single sample. The kit performance has been optimized for analysis of specific cytokines in tissue culture supernatants, EDTA plasma, and serum samples from rhesus, cynomolgus, baboon, or pigtailed macaque non-human primate species. In this kit, the antibodies are human-specific and the standards are recombinant human proteins. The antibodies are cross-reactive with non-human primate cytokines. Even though this kit uses recombinant human proteins and human-specific antibodies for the measurement of NHP proteins, measured relative values are reported in pg/mL. The kit provides sufficient reagents for 80 tests.

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 the 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

Six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for human IL-2, IL-4, IL-5, IL-6, TNF, and IFN-γ proteins. The six bead populations are mixed together to form the bead array that is resolved in a red channel of a flow cytometer (see Figure 1).

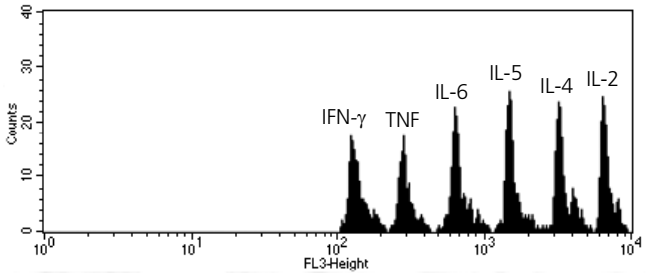


Figure 1

During the assay procedure, you will mix the capture beads with the recombinant standards or unknown samples and incubate them with the human-specific, 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 BD CBA assays 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-sixth the quantity necessary for conventional ELISA assays due to the detection of six 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 six conventional ELISAs.

Limitations

Assay Limitations

The theoretical limit of detection of the BD CBA NHP 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 in a given experiment may vary slightly. See Theoretical limit of detection (page 30) and Precision (page 44).

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).

The use of recombinant human protein standards and human-specific antibodies to quantitate NHP proteins yields relative values only, even though they are still being reported as pg/mL.

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

This kit contains the following components sufficient for 80 tests.

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-5 Capture Beads	1 vial, 0.8 mL
A4	Human IL-6 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
B	Non-Human Primate Th1/Th2 PE Detection Reagent	1 vial, 4 mL
C	Human Th1/Th2 Cytokine Standards	2 vials lyophilized
D	Cytometer Setup Beads	1 vial, 1.5 mL

Vial label	Reagent	Quantity
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

Human Cytokine Capture Beads (A1–A6): An 80-test vial of each specific capture bead (A1–A6). The specific capture beads, having discrete fluorescence intensity characteristics, are distributed from brightest (A1) to dimmest (A6).

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 per test.

Antibody and standard reagents

Non-Human Primate Th1/Th2 PE Detection Reagent (B): An 80-test vial of PE-conjugated anti-human IL-2, IL-4, IL-5, IL-6, TNF, and IFN-γ antibodies, formulated for use at 50 µL per test.

Human Th1/Th2 Cytokine Standards (C): Two vials containing lyophilized recombinant human cytokine proteins. Each vial is 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 formulated for use at 50 µL per 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 per test. This reagent is used with the Cytometer Setup Beads to set the initial instrument compensation settings.

Buffer reagents

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.

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

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–A6, 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 discharging to avoid accumulation of potentially explosive deposits in plumbing.

Human Th1/Th2 Cytokine Standards (component 51-2428KC) contains 0.02% (w/w) of a CMIT/MIT mixture (3:1), which is a mixture of: 5-chloro-2-methyl-4-isothiazolin-3-one [EC No 247-500-7] and 2-methyl-4-isothiazolin-3-one [EC No 220-239-6] (3:1).

Hazard statements	May cause an allergic skin reaction.
Precautionary statements	Wear protective gloves / eye protection. Wear protective clothing. Avoid breathing mist/vapours/spray. If skin irritation or rash occurs: Get medical advice/ attention. IF ON SKIN: Wash with plenty of water. Dispose of contents/container in accordance with local/ regional/national/international regulations.

2

Before you begin

This section covers the following topics:

- Workflow overview (page 14)
- Required materials (page 15)

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Workflow overview

Workflow

The overall workflow consists of the following steps.

Step	Description
1	Preparing Human Th1/Th2 Cytokine Standards (page 18)
2	Mixing NHP 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 Non-Human Primate Th1/Th2 Cytokine Assay (page 24)
6	Acquiring samples (instructions can be found at bdbiosciences.com/cbasetup)
7	Data analysis (page 27)

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	3 hours

Required materials

Materials required but not provided

In addition to the reagents provided in the BD CBA NHP 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 channels
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
Note: Visit bdbiosciences.com/cbasetup for setup protocols.		

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

3

Assay preparation

This section covers the following topics:

- Preparing Human Th1/Th2 Cytokine Standards (page 18)
- Mixing NHP Th1/Th2 Cytokine Capture Beads (page 20)
- Diluting samples (page 21)

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Preparing Human Th1/Th2 Cytokine Standards

Purpose of this procedure

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

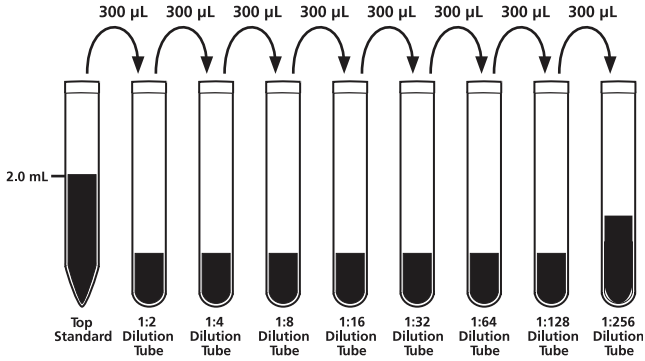
You must prepare fresh cytokine standards to run with each experiment. Do not store or reuse reconstituted or diluted standards.

Procedure

To reconstitute and serially dilute the standards:

1. Open one vial of lyophilized Human Th1/Th2 Standards. Transfer the standard spheres to a 15-mL 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 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 remaining 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. Do not vortex.

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 Assay Diluent to serve as the 0-pg/mL negative control.

Concentration of standards

See Performing the Non-Human Primate Th1/Th2 Cytokine Assay (page 24) for a listing of the concentration (pg/mL) of all six recombinant proteins in each standard.

Next step

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

Mixing NHP Th1/Th2 Cytokine Capture Beads

Purpose of this procedure

The Capture Beads are bottled individually (A1–A6). You must pool all six 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) that are required for the experiment (for example, 8 unknowns + 9 cytokine standard dilutions + 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. 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 Non-Human Primate Th1/Th2 Cytokine Assay (page 24). If you need to dilute samples having high-protein 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 the 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 Non-Human Primate Th1/Th2 Cytokine Assay (page 24), and you can perform instrument setup during the 3-hour staining incubation.

4

Assay procedure

This section covers the following topics:

- Performing the Non-Human Primate Th1/Th2 Cytokine Assay (page 24)
- Data analysis (page 27)

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Performing the Non-Human Primate Th1/Th2 Cytokine Assay

Before you begin

- Prepare the standards as described in Preparing Human Th1/Th2 Cytokine Standards (page 18).
- Mix the Capture Beads as described in Mixing NHP 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 Human Th1/Th2 Cytokine Standard dilutions to the control assay 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	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 tubes.

4. Add 50 µL of the NHP Th1/Th2 PE Detection Reagent to the 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.
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)
   - NHP 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 3 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.

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 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 27).

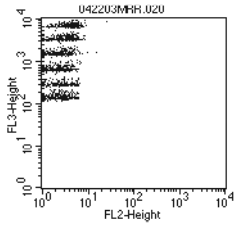
Data analysis

How to analyze

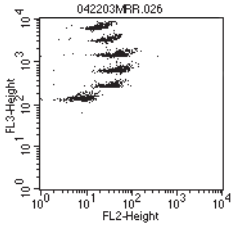
Analyze BD CBA Non-Human Primate Th1/Th2 Cytokine Kit data using FCAP Array software. For instructions on analysis, go to bdbiosciences.com/cbasetup and see the *Guide to Analyzing Data from BD CBA Kits Using FCAP Array Software*.

Typical data

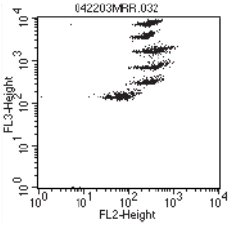
The following data, acquired using BD CellQuest™ software, shows standards and detectors alone.



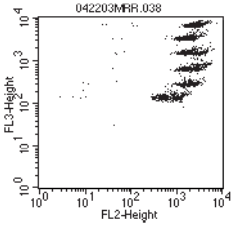
Negative control: 0 pg/mL



Standard: 80 pg/mL



Standard: 625 pg/mL

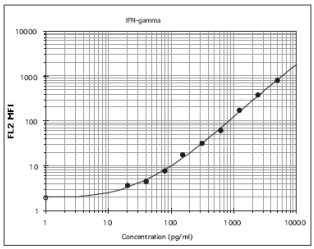


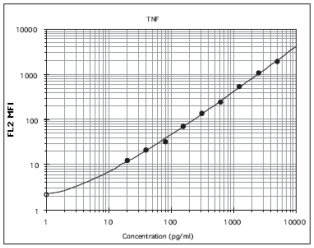
Standard: 5,000 pg/mL

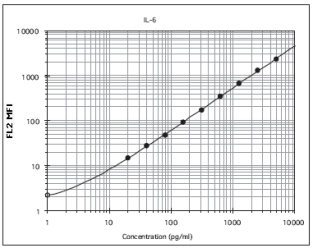
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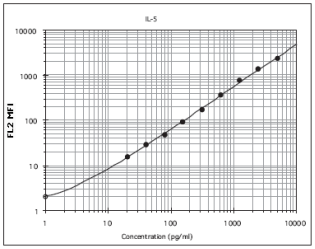
Standard curve examples

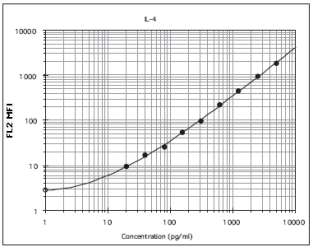
The following graphs represent standard curves from the BD CBA Non-Human Primate Th1/Th2 Cytokine Standards.

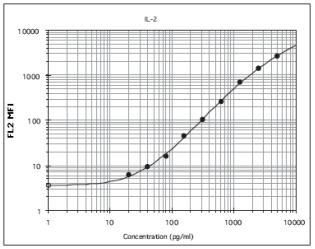












5

Performance

This section covers the following topics:

- Theoretical limit of detection (page 30)
- Recovery (page 31)
- Linearity (page 37)
- Specificity (page 43)
- Precision (page 44)

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Theoretical limit of detection

Experiment details

The individual standard curve range for a given cytokine defines the minimum and maximum quantifiable levels (ie, 20 pg/mL and 5,000 pg/mL) using the BD CBA NHP Th1/Th2 Cytokine Kit. By applying the four-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 NHP 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	3.5	0.2	3.6
IL-4	2.8	0.1	0.9
IL-5	2.1	0.1	0.3
IL-6	2.1	0.1	0.1
TNF	2.2	0.2	0.4
IFN-γ	1.9	0.1	3.3

Recovery

Experiment details

The individual cytokine proteins were spiked into various cell culture and non-human primate matrices at three different levels within the assay range. The matrices used in these experiments were not diluted before addition of the cytokine protein. The plasma samples in these experiments were EDTA treated. Results are compared with the same concentrations of the cytokines spiked in the Assay Diluent.

Recovery data

Pooled rhesus plasma (n=4)

Protein	Spike conc. (pg/mL)	Observed conc. (pg/mL)	% Recovery
IFN-γ	80 625 2,500	58 375 1,466	73% 60% 59%
TNF	80 625 2,500	73 443 1,875	91% 71% 75%
IL-6	80 625 2,500	68 376 1,697	85% 60% 68%
IL-5	80 625 2,500	82 543 2,354	102% 87% 94%
IL-4	80 625 2,500	35 269 1,527	44% 43% 61%
IL-2	80 625 2,500	71 483 4,020	88% 77% 82%

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Pooled rhesus sera (n=4)

Protein	Spike conc. (pg/mL)	Observed conc. (pg/mL)	% Recovery
IFN-γ	80 625 2,500	66 318 1,245	82% 51% 50%
TNF	80 625 2,500	86 493 2,123	107% 79% 85%
IL-6	80 625 2,500	78 448 1,875	97% 72% 75%
IL-5	80 625 2,500	91 514 2,171	114% 82% 87%
IL-4	80 625 2,500	42 279 1,433	52% 45% 57%
IL-2	80 625 2,500	72 417 1,868	91% 67% 75%

Pooled baboon plasma (n=4)

Protein	Spike conc. (pg/mL)	Observed conc. (pg/mL)	% Recovery
IFN-γ	80 625 2,500	70 385 1,475	88% 62% 59%
TNF	80 625 2,500	80 520 2,306	100% 83% 92%
IL-6	80 625 2,500	82 524 2,209	103% 84% 88%

Protein	Spike conc. (pg/mL)	Observed conc. (pg/mL)	% Recovery
IL-5	80 625 2,500	82 598 2,822	102% 96% 113%
IL-4	80 625 2,500	83 550 2,315	104% 88% 93%
IL-2	80 625 2,500	66 551 2,412	83% 88% 96%

Pooled baboon sera (n=4)

Protein	Spike conc. (pg/mL)	Observed conc. (pg/mL)	% Recovery
IFN-γ	80 625 2,500	59 355 1,152	73% 57% 46%
TNF	80 625 2,500	69 487 1,860	86% 78% 74%
IL-6	80 625 2,500	66 466 1,607	83% 75% 64%
IL-5	80 625 2,500	61 458 2,090	76% 73% 84%
IL-4	80 625 2,500	59 459 1,801	74% 73% 72%
IL-2	80 625 2,500	65 527 2,127	82% 84% 85%

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Pooled cynomolgus plasma (n=4)

Protein	Spike conc. (pg/mL)	Observed conc. (pg/mL)	% Recovery
IFN-γ	80 625 2,500	38 370 1,468	48% 59% 59%
TNF	80 625 2,500	46 375 1,726	57% 60% 69%
IL-6	80 625 2,500	57 444 1,917	71% 71% 77%
IL-5	80 625 2,500	59 492 2,179	74% 79% 87%
IL-4	80 625 2,500	47 419 1,852	59% 67% 74%
IL-2	80 625 2,500	27 263 1,285	34% 42% 51%

Pooled cynomolgus sera (n=4)

Protein	Spike conc. (pg/mL)	Observed conc. (pg/mL)	% Recovery
IFN-γ	80 625 2,500	50 407 1,698	62% 65% 68%
TNF	80 625 2,500	51 388 1,824	63% 62% 73%
IL-6	80 625 2,500	57 419 1,844	71% 67% 74%

Protein	Spike conc. (pg/mL)	Observed conc. (pg/mL)	% Recovery
IL-5	80 625 2,500	61 494 2,246	76% 79% 90%
IL-4	80 625 2,500	61 482 2,119	76% 77% 85%
IL-2	80 625 2,500	49 404 1,939	61% 65% 78%

Pooled pigtailed plasma (n=4)

Protein	Spike conc. (pg/mL)	Observed conc. (pg/mL)	% Recovery
IFN-γ	80 625 2,500	75 574 1,875	94% 92% 75%
TNF	80 625 2,500	69 553 2,284	87% 89% 91%
IL-6	80 625 2,500	79 574 2,256	99% 92% 90%
IL-5	80 625 2,500	74 616 2,408	92% 98% 96%
IL-4	80 625 2,500	75 621 2,469	94% 99% 99%
IL-2	80 625 2,500	63 542 2,226	78% 87% 89%

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Pooled pigtailed sera (n=4)

Protein	Spike conc. (pg/mL)	Observed conc. (pg/mL)	% Recovery
IFN-γ	80 625 2,500	85 605 2,053	106% 97% 82%
TNF	80 625 2,500	70 579 2,487	88% 93% 99%
IL-6	80 625 2,500	81 583 2,425	101% 93% 97%
IL-5	80 625 2,500	73 651 2,587	91% 104% 103%
IL-4	80 625 2,500	57 514 2,238	72% 82% 90%
IL-2	80 625 2,500	57 514 2,183	71% 82% 87%

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Cell culture media

Protein	Spike conc. (pg/mL)	Observed conc. (pg/mL)	% Recovery
IFN-γ	80 625 2,500	111 541 2,352	139% 87% 94%
TNF	80 625 2,500	85 497 2,632	106% 80% 105%
IL-6	80 625 2,500	84 480 2,331	105% 77% 93%
IL-5	80 625 2,500	77 495 2,441	97% 79% 98%
IL-4	80 625 2,500	84 499 2,323	104% 78% 93%
IL-2	80 625 2,500	88 521 2,572	110% 83% 103%

Linearity

Experiment details

In two experiments, the following cell culture and non-human primate matrices were spiked with IL-2, IL-4, IL-5, IL-6, TNF, and IFN-γ and serially diluted with Assay Diluent.

Linearity data

Pooled rhesus serum averages (n=4)

Dilution	Observed (pg/mL)					
	IFN-γ	TNF	IL-6	IL-5	IL-4	IL-2
Neat	2,675.5	4,485.6	4,368.5	5,455.5	4,163.9	4,340.3
1:2	1,825.8	2,574.9	2,424.5	2,771.7	2,112.9	2,352.0
1:4	1,098.9	1,321.7	1,258.4	1,442.3	1,022.9	1,214.2
1:8	542.6	625.1	598.1	659.9	564.6	576.8
1:16	269.4	319.5	306.1	319.9	288.6	295.9
1:32	105.5	154.9	148.9	156.9	132.4	148.3
1:64	47.4	79.3	74.6	79.9	71.6	77.9
1:128	20.6	36.6	34.2	33.7	34.1	31.9
1:256	11.8	19.9	18.3	19.3	16.2	15.2
Slope	1.071	1.006	1.014	1.052	0.994	1.019

Pooled rhesus plasma averages (n=4)

Dilution	Observed (pg/mL)					
	IFN-γ	TNF	IL-6	IL-5	IL-4	IL-2
Neat	3,414.7	3,960.6	3,888.7	4,491.4	3,684.9	4,227.8
1:2	1,894.4	2,311.3	2,248.1	2,515.3	1,828.8	2,292.4
1:4	912.2	1,255.7	1,194.0	1,330.8	950.8	1,159.0
1:8	475.8	608.9	586.1	650.8	512.8	563.5
1:16	234.5	306.2	289.3	307.9	264.8	287.7
1:32	97.2	152.4	150.1	161.7	130.6	140.5
1:64	44.6	77.8	72.2	79.4	68.5	67.8
1:128	19.2	42.0	38.9	40.7	31.5	29.0
1:256	9.0	21.3	19.9	19.4	15.2	14.6
Slope	1.136	0.967	0.978	0.999	0.980	1.030

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Pooled baboon serum averages (n=4)

Dilution	Observed (pg/mL)					
	IFN-γ	TNF	IL-6	IL-5	IL-4	IL-2
Neat	1,507.9	3,283.3	3,788.4	4,622.7	3,884.5	3,669.6
1:2	988.4	2,078.2	2,110.1	2,744.9	2,222.8	2,701.9
1:4	581.6	1,102.9	1,086.4	1,435.8	1,084.6	1,353.7
1:8	368.5	587.8	574.6	674.7	584.3	704.8
1:16	212.9	315	290.1	321.2	281.2	309.6
1:32	115.1	149.8	134.7	150.9	140.2	145.6
1:64	46	60	54.1	53.6	58.3	55
1:128	23.7	37.9	35.8	36.9	531.9	28.8
1:256	11.9	18	16.65	16.5	14.6	11.6
Slope	0.938	0.962	1.043	1.047	1.018	1.073

Pooled baboon plasma averages (n=4)

Dilution	Observed (pg/mL)					
	IFN-γ	TNF	IL-6	IL-5	IL-4	IL-2
Neat	2,052.6	3,247.8	3,592.7	3,728.9	3,588.5	4,314.7
1:2	1,206.7	1,463.8	1,608	1,709	1,945.7	2,280.5
1:4	678.5	920.2	853.2	805	861.4	1,100.8
1:8	389.3	472.4	452.2	457	506.2	596.1
1:16	238.9	314.9	287.5	293	145.1	287.3
1:32	133.8	141.5	135.3	136.2	131.8	137.2
1:64	70.7	72.7	68.7	66.4	57.5	54.2
1:128	26.2	28.6	28.3	25.5	23.7	20.8
1:256	18.4	17.4	16.6	15.4	14.5	15
Slope	0.864	0.950	1.007	0.994	1.011	1.145

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Pooled cynomolgus serum averages (n=4)

	Observed (pg/mL)					
Dilution	**IFN-γ**	**TNF**	**IL-6**	**IL-5**	**IL-4**	**IL-2**
Neat	3,626.0	3,941.9	4,502.2	3,792.8	4,456.1	4,395.3
1:2	2,048.9	2,175.0	2,297.4	2,634.3	2,297.9	2,356.5
1:4	1,048.8	1,061.2	1,122.2	1,361.1	1,019.1	1,053.4
1:8	651.2	580.4	562.6	592.9	608.5	553.6
1:16	384.0	296.7	290.2	294.1	312.0	286.9
1:32	232.9	148.3	148.0	153.4	175.0	163.3
1:64	126.7	77.4	75.7	75.5	91.1	87.6
1:128	104.5	42.2	41.3	36.8	39.2	34.7
1:256	39.3	20.8	20.1	22.2	22.5	20.0
Slope	0.903	0.973	1.006	0.995	1.051	1.107

Pooled cynomolgus plasma averages (n=4)

	Observed (pg/mL)					
Dilution	**IFN-γ**	**TNF**	**IL-6**	**IL-5**	**IL-4**	**IL-2**
Neat	2,953.1	4,068.3	4,464.2	4,842.9	4,268.7	4,580.1
1:2	1,805.5	2,273.0	2,301.2	2,340.1	2,280.0	2,126.3
1:4	893.5	1,127.1	1,119.1	1,183.8	1,111.4	976.3
1:8	448.6	530.1	494.0	518.9	573.5	497.4
1:16	256.6	286.8	270.1	285.3	309.7	249.6
1:32	117.0	133.0	130.2	137.7	159.3	120.3
1:64	71.3	71.3	65.7	67.8	74.3	55.0
1:128	34.2	33.7	31.9	33.0	27.2	25.9
1:256	42.1	16.9	16.3	16.4	12.8	14.9
Slope	1.154	1.026	1.052	1.045	1.117	1.219

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Pooled pigtailed serum averages (n=4)

Dilution	Observed (pg/mL)					
	IFN-γ	TNF	IL-6	IL-5	IL-4	IL-2
Neat	2,379.4	2,580.9	1,960.1	2,991.2	2,365.7	2,681.4
1:2	1,356.9	1,415.8	1,381.7	1,549.8	1,241.0	1,396.9
1:4	696.3	757.4	713.9	832.8	682.6	699.3
1:8	353.4	389.1	352.0	391.0	354.4	338.2
1:16	155.3	178.7	172.8	181.2	160.8	157.2
1:32	81.2	96.3	88.0	93.5	82.5	83.2
1:64	39.1	48.3	44.8	43.2	36.1	37.2
1:128	17.8	19.3	18.0	18.0	15.4	16.7
1:256	11.0	9.4	8.5	7.6	12.8	9.6
Slope	1.210	1.068	1.194	0.956	0.995	1.261

Pooled pigtailed plasma averages (n=4)

Dilution	Observed (pg/mL)					
	IFN-γ	TNF	IL-6	IL-5	IL-4	IL-2
Neat	1,975.4	2,310.8	2,432.5	2,590.5	2,482.9	2,455.3
1:2	947.8	1,210.0	1,089.6	1,293.6	1,241.8	1,142.2
1:4	524.2	679.1	632.9	733.3	631.7	635.2
1:8	276.3	344.3	327.2	350.1	314.1	325.1
1:16	134.2	176.5	167.2	176.8	151.3	155.2
1:32	55.2	79.8	77.3	77.1	71.7	74.4
1:64	38.5	39.7	36.4	37.8	29.4	39.1
1:128	31.5	23.1	21.6	22.5	31.0	28.4
1:256	10.3	10.2	9.1	10.1	13.0	9.9
Slope	1.002	1.011	1.108	1.029	0.975	1.123

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Cell culture medium averages (n=4)

Dilution	Observed (pg/mL)					
	IFN-γ	TNF	IL-6	IL-5	IL-4	IL-2
Neat	3,985.4	4,342.5	4,040.1	4,324.4	4,386.9	4,558.0
1:2	1,963.6	2,387.1	2,359.1	2,334.2	2,169.2	2,252.1
1:4	970.5	1,127.8	1,144.2	1,172.4	1,025.2	1,068.1
1:8	533.9	635.6	601.6	638.6	539.6	583.4
1:16	253.8	325.7	319.9	321.4	272.1	287.0
1:32	102.2	124.7	109.8	111.4	113.5	128.1
1:64	58.1	77.8	76.6	76.3	67.2	66.8
1:128	27.9	38.7	36.0	36.2	31.5	31.8
1:256	13.1	17.6	17.8	16.7	16.0	14.9
Slope	0.994	1.021	1.014	1.028	1.034	1.083

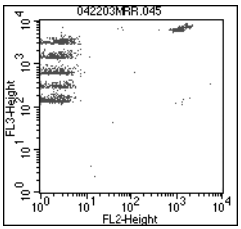
Specificity

Experiment details

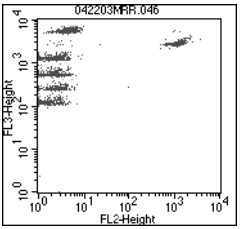
The antibody pairs used in the BD CBA NHP Th1/Th2 Cytokine Kit have been screened for specific reactivity with their specific cytokines. Analysis of samples containing only a single recombinant cytokine protein found no cross-reactivity or background detection of cytokine in other Capture Bead populations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

Specificity data

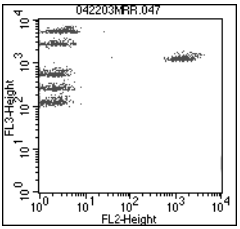
Data for the detection of individual proteins was analyzed using BD CellQuest software.



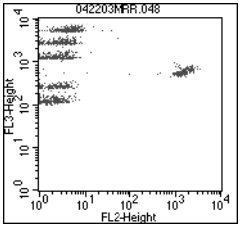
IL-2



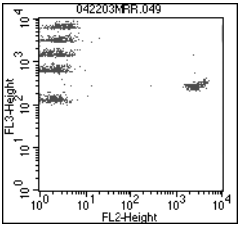
IL-4



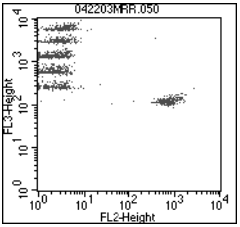
IL-5



IL-6



TNF



IFN-γ

Precision

Intra-assay precision

Ten replicates of each of three different levels (80, 625, and 2,500 pg/mL) of IL-2, IL-4, IL-5, IL-6, TNF, and IFN-γ were tested.

Protein	Actual mean conc. (pg/mL)	SD	% CV
IL-2	65 551 2,303	6 49 112	8.6% 8.9% 4.9%
IL-4	69 562 2,303	5 49 142	7.2% 8.8% 6.2%
IL-5	73 551 2,231	10 84 154	13.8% 15.3% 6.9%
IL-6	75 576 2,534	10 73 150	13.2% 12.7% 5.9%
TNF	77 575 2,545	7 75 156	9.2% 13.0% 6.1%
IFN-γ	68 500 2,058	7 61 164	10.6% 12.2% 8.0%

Inter-assay precision

Three different levels (80, 625, and 2,500 pg/mL) of IL-2, IL-4, IL-5, IL-6, TNF, and IFN-γ were tested in four experiments conducted by different operators.

Note: Eight replicates (total number of assay tubes) were tested for each concentration of protein.

Protein	Actual mean conc. (pg/mL)	SD	% CV
IL-2	75 683 2,432	12 99 186	15.7% 14.5% 7.6%
IL-4	73 628 2,412	8 73 138	11.2% 11.6% 5.7%
IL-5	77 705 2,504	7 121 173	9.1% 17.2% 6.9%
IL-6	80 696 2,594	7 110 183	9.0% 15.8% 7.0%
TNF	77 673 2,499	7 106 159	9.1% 15.7% 6.4%
IFN-γ	75 628 2,348	11 93 194	14.3% 14.8% 8.3%

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6

Reference

This section covers the following topics:

- Troubleshooting (page 48)
- References (page 50)

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Troubleshooting

Recommended actions

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

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

Problem	Recommended action
Variation between duplicate samples	Vortex the Capture Beads before pipetting. Beads can aggregate.
Low bead number in samples	• Avoid aspiration of beads during the wash step. • Do not wash or resuspend beads in volumes higher than the recommended volumes.
High background	• Test various sample dilutions. The sample may be too concentrated. • Remove excess NHP 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	The 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.

Problem	Recommended action
Debris (FSC/SSC) during sample acquisition	• Increase the FSC threshold or further dilute the samples. • Increase the number of wash steps, if necessary. • Make a tighter FSC/SSC gate around the bead population. • Centrifuge or filter samples to reduce debris before staining samples with the BD CBA Non-Human Primate Th1/Th2 Cytokine Kit.
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.
Standards assay tubes show low fluorescence or a poor standard curve	• Ensure that all components are properly prepared and stored. • Use a new vial of standards with each experiment, and once reconstituted, do not use after 12 hours. • Ensure that incubation times were appropriate.
All samples are positive or above the high standard median 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 acquiring on the flow cytometer. However, this may affect assay performance and should be validated.

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