BD™ Cytometric Bead Array (CBA)

Human Th1/Th2 Cytokine Kit

Instruction Manual

Cat. No. 550749

BD

Kit Contents

80 Tests (50 samples and 2 standard curves)

(Store the following items at 4°C)

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-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

B Human Th1/Th2 PE* Detection Reagent: 1 vial, 4 ml

C Human 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

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

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Introduction

Flow cytometry is an analysis tool that allows for the discrimination of different particles on the basis of size and color. Multiplexing is the simultaneous assay of many analytes in a single sample. The BD™ Cytometric Bead Array (CBA) employs a series of particles with discrete fluorescence intensities to simultaneously detect multiple soluble analytes. The BD CBA is combined with flow cytometry to create a powerful multiplexed assay.

The BD CBA system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. Each bead in a BD CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate. The BD CBA capture bead mixture is in suspension to allow for the detection of multiple analytes in a small volume sample. The combined advantages of the broad dynamic range of fluorescent detection via flow cytometry and the efficient capturing of analytes via suspended particles enable BD CBA to use fewer sample dilutions and to obtain the value of an unknown in substantially less time (compared to conventional ELISA).

The BD CBA Human Th1/Th2 Cytokine Kit can be used to quantitatively measure Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-10 (IL-10), 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.

Principle of the Test

Six bead populations with distinct fluorescence intensities have been coated with capture antibodies specific for IL-2, IL-4, IL-5, IL-10, TNF, and IFN-γ proteins. The six bead populations are mixed together to form the BD CBA that is resolved in a red channel (ie, FL3 or FL4) of a flow cytometer.

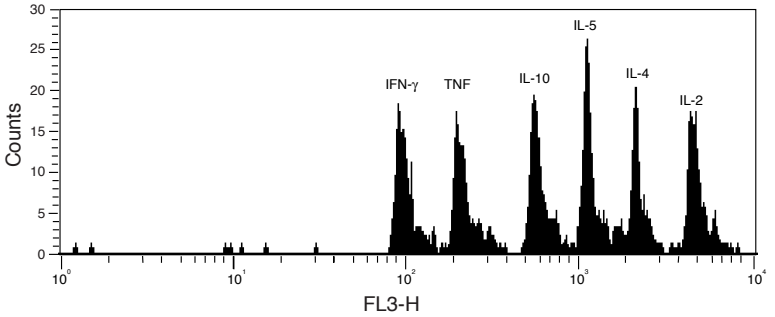


Figure 1

The cytokine capture beads are mixed with the PE-conjugated detection antibodies and then incubated with recombinant standards or test samples to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results are generated in graphical and tabular format using the BD CBA Analysis Software or FCAP Array™ Software. The kit provides sufficient reagents for the quantitative analysis of 50 test samples and the generation of two standard curve sets.

Advantages

The BD CBA provides several advantages when compared with 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

The theoretical limit of detection of the BD CBA Human 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*** and ***Precision*** information on pages 25 and 29 respectively).

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™ 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.

Reagents Provided

Bead Reagents

Human Cytokine Capture Beads (A1 – A6): The specific capture beads, having discrete fluorescence intensity characteristics, are distributed from brightest to dimmest as follows:

Bead	Specificity
(Brightest) A1	IL-2
A2	IL-4
A3	IL-5
A4	IL-10
A5	TNF
(Dimmest) A6	IFN-γ

A single 80-test vial of each specific capture bead (A1 – A6) is included in this kit. Store at 4°C. Do not freeze.

Note: The antibody-conjugated beads will settle out of suspension over time. It is necessary to vortex the vial vigorously for 3 – 5 seconds before taking a bead suspension aliquot.

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 PE Detection Reagent (B): An 80-test vial of PE-conjugated anti-human IL-2, IL-4, IL-5, IL-10, TNF and IFN-γ antibodies that is formulated for use at 50 µl/test. Store at 4°C. Do not freeze.

Human Th1/Th2 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. Store at 4°C.

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. Store at 4°C. Do not freeze.

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. Store at 4°C. Do not freeze.

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. Store at 4°C.

Assay Diluent (G): A single, 30 ml bottle of a buffered protein* solution (1×) used to reconstitute and dilute the Human Th1/Th2 Cytokine Standards and to dilute test samples. Store at 4°C.

Serum Enhancement Buffer (H): A single, 10 ml bottle of a buffered protein* solution (1×) used to dilute mixed Capture Beads when testing serum or plasma samples. Store at 4°C.

Warnings and Precautions

Hazardous Ingredients:

Sodium Azide:

Components A1 - A6, B, D, E1 - E2, F, G, and H contain 0.09% sodium azide. Sodium azide yields a highly toxic hydrazoic acid under acidic conditions. Avoid exposure to skin and eyes, ingestion, and contact with heat, acids, and metals. Wash exposed skin with soap and water. Flush eyes with water. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

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

Materials Required but not Provided

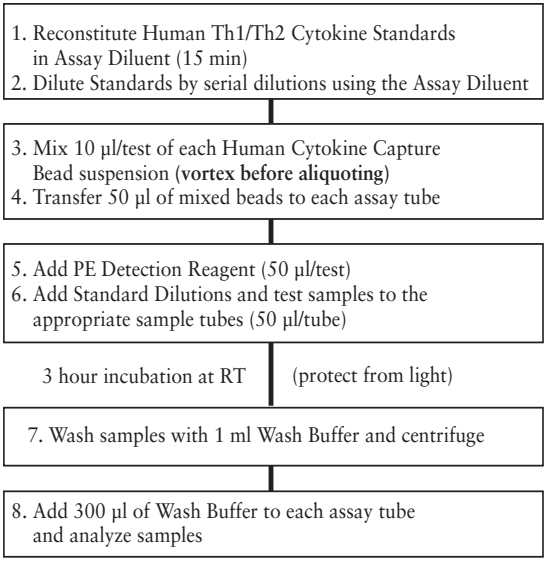
In addition to the reagents provided in the BD CBA Human Th1/Th2 Cytokine Kit, the following items are also required:

- A flow cytometer equipped with a 488 nm laser capable of detecting and distinguishing fluorescence emissions at 576 and 670 nm (eg, BD FACScan™ or BD FACSCalibur™ instruments) and BD CellQuest™ or BD CellQuest Pro Software.
- 12 × 75 mm sample acquisition tubes for a flow cytometer (eg, BD Falcon™ Cat. No. 352008.)
- BD CBA Software, or FCAP Array software (Cat. No. 641488).

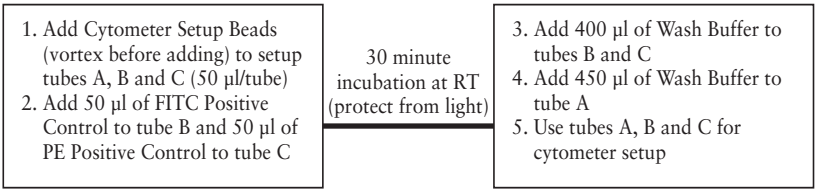
  Note: BD CBA Software is no longer available for purchase but is still supported for current users on existing compatible systems.
- BD Calibrite™ 3 Beads, (Cat. No. 340486).

Overview: BD CBA Human Th1/Th2 Cytokine Kit Assay Procedure

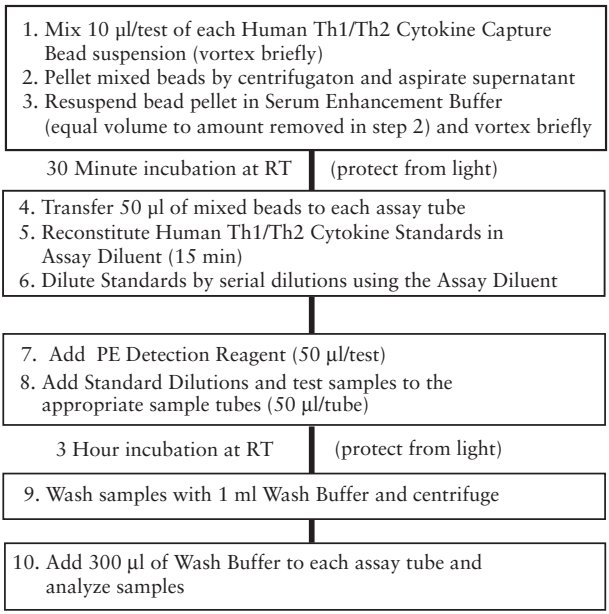
Culture Supernatant Assay Procedure



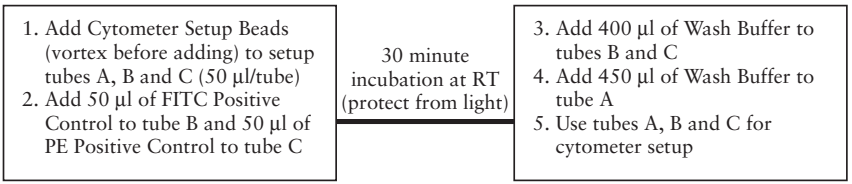
Cytometer Setup Bead Procedure



Serum/Plasma Assay Procedure



Cytometer Setup Bead Procedure



Preparation of Human Th1/Th2 Cytokine Standards

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

1. Open one vial of lyophilized Human Th1/Th2 Standards. Transfer the standard spheres to a polypropylene tube (eg, 15 ml Conical Tube, BD Falcon Cat. No. 352097). Label tube "Top Standard".
2. Reconstitute the standards with 2.0 ml of Assay Diluent. Allow the reconstituted standard to equilibrate for at least 15 minutes before making dilutions. **Mix reconstituted protein by pipette only. Do not vortex or mix vigorously.**
3. Label 12 × 75 mm tubes (BD Falcon Cat. No. 352008) 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 to each of the remaining tubes.
5. Perform a serial dilution by transferring 300 µl from the Top Standard to the 1:2 dilution tube and mix thoroughly. 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 and mix thoroughly (see ***Figure 2***). **Mix by pipette only, do not vortex.** Prepare one tube containing Assay Diluent to serve as the 0 pg/ml negative control.

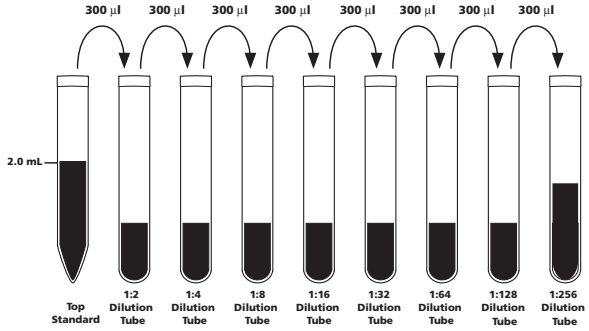


Figure 2. Preparation of Human Th1/Th2 Cytokine Standard Dilutions

The approximate concentration (pg/ml) of recombinant protein in each dilution tube is shown in ***Table 1***.

Table 1. Human Th1/Th2 Cytokine Standard concentrations after dilution

Protein (pg/ml)	Top Standard	1:2 Dilution Tube	1:4 Dilution Tube	1:8 Dilution Tube	1:16 Dilution Tube	1:32 Dilution Tube	1:64 Dilution Tube	1:128 Dilution Tube	1:256 Dilution Tube
Human IL-2	5000	2500	1250	625	312.5	156	80	40	20
Human IL-4	5000	2500	1250	625	312.5	156	80	40	20
Human IL-5	5000	2500	1250	625	312.5	156	80	40	20
Human IL-10	5000	2500	1250	625	312.5	156	80	40	20
Human TNF	5000	2500	1250	625	312.5	156	80	40	20
Human IFN-γ	5000	2500	1250	625	312.5	156	80	40	20

Preparation of Mixed Human Th1/Th2 Cytokine Capture Beads

The Capture Beads are bottled individually and it is necessary to pool the bead reagents (A1 – A6) immediately before mixing them together with the PE Detection Reagent, standards and samples. It is recommended that this procedure be used for preparing the mixed Human Th1/Th2 Cytokine Capture Beads for experiments in which cell culture supernatant samples will be analyzed. For experiments testing serum or plasma samples, refer to ***Preparation of Mixed Human Th1/Th2 Cytokine Capture Beads for Serum and Plasma Sample Analysis***, page 14.

1. Determine the number of assay tubes (including standards and controls) that are 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 a few seconds before mixing.
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.

The mixed Capture Beads are now ready to be transferred to the assay tubes (50 µl of mixed Capture Beads/tube) as described in ***BD CBA Human Th1/Th2 Cytokine Kit Assay Procedure***, page 15.

Note: Discard excess mixed Capture Beads. Do not store after mixing.

Preparation of Mixed Human Th1/Th2 Cytokine Capture Beads for Serum and Plasma Sample Analysis

It is recommended that the following procedure be followed for preparing the mixed Human Th1/Th2 Cytokine Capture Beads for experiments in which serum and plasma samples will be analyzed. Use of this procedure will reduce the chances of false-positive results due to the effects of serum or plasma proteins. This procedure may also be used with cell culture supernatant samples.

1. Follow steps 1 – 4 under Preparation of Mixed Human Th1/Th2 Cytokine Capture Beads, page 13.
2. Centrifuge mixed Capture Beads at 200 × g for 5 minutes .
3. Carefully aspirate and discard the supernatant.
4. Resuspend the mixed Capture Beads pellet in Serum Enhancement Buffer (equal volume to amount removed in step 3) and vortex thoroughly.
5. Incubate the mixed Capture Beads for 30 minutes at RT and protect from direct exposure to light.

The mixed Capture Beads are now ready to be transferred to the assay tubes (50 µl of mixed Capture Beads/tube) as described in ***BD CBA Human Th1/Th2 Cytokine Kit Assay Procedures***, page 15.

Note: Discard excess mixed Capture Beads. Do not store after mixing.

Preparation of Test Samples

The standard curve for each cytokine covers a defined set of concentrations from 20 – 5000 pg/ml. It may be necessary to dilute test samples to ensure that their mean fluorescence values fall within the limits or range of the generated cytokine standard curve. For best results, samples that are known or assumed to contain high levels of a given cytokine should be diluted as described below.

1. Dilute test 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 before transferring samples to the appropriate assay tubes containing mixed Capture Beads and PE Detection Reagent.

BD CBA Human Th1/Th2 Cytokine Kit Assay Procedure

Following the preparation and dilution of the standards and mixing of the capture beads, transfer these reagents and test samples to the appropriate assay tubes for incubation and analysis. The serum/plasma assay procedure (page 16) should be used for any experiment testing serum or plasma samples. The serum/plasma assay procedure also works for culture supernatant. **In order to calibrate the flow cytometer and quantitate test samples, it is necessary to run the Cytokine Standards and the Cytometer Setup controls in each experiment.** See ***Table 2*** for a detailed description of the reagents added to the Cytokine Standard control assay tubes. The Cytometer Setup procedure is described on page 17.

Culture Supernatant Assay Procedure

1. Add 50 µl of the mixed Capture Beads (prepared using the procedure described in ***Preparation of Mixed Human Th1/Th2 Cytokine Capture Beads***, page 13) to the appropriate assay tubes. Vortex the mixed Capture Beads before adding to the assay tubes.
2. Add 50 µl of the Human Th1/Th2 PE Detection Reagent to the assay tubes.
3. Add 50 µl of the Human Th1/Th2 Cytokine Standard dilutions to the control assay tubes.
4. Add 50 µl of each test sample to the test assay tubes.
5. Incubate the assay tubes for 3 hours at RT and protect from direct exposure to light. During this incubation, perform the Cytometer Setup procedure described on pages 17 – 18.
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.
9. Begin analyzing samples on a flow cytometer. **Vortex each sample for 3 – 5 seconds immediately before analyzing on the flow cytometer.**

   Note: It is necessary to analyze CBA samples on the day of the experiment. Prolonged storage of samples, once the assay is complete, can lead to increased background and reduced sensitivity.

Serum/Plasma Assay Procedure

1. Add 50 µl of the mixed Capture Beads (prepared using the procedure described in ***Preparation of Mixed Human Th1/Th2 Cytokine Capture Beads for Serum and Plasma Sample Analysis***, page 14) to the appropriate assay tubes. Vortex the mixed Capture Beads before adding to the assay tubes.
2. Add 50 µl of the Human Th1/Th2 PE Detection Reagent to the assay tubes.
3. Add 50 µl of the Human Th1/Th2 Cytokine Standard dilutions to the control assay tubes.
4. Add 50 µl of each test sample to the test assay tubes.
5. Incubate the assay tubes for 3 hours at RT and protect from direct exposure to light. During this incubation, perform the Cytometer Setup procedure described on pages 17 – 18.
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.
9. Begin analyzing samples on a flow cytometer. **Vortex each sample for 3 – 5 seconds immediately before analyzing on the flow cytometer.**

   Note: It is necessary to analyze CBA samples on the day of the experiment. Prolonged storage of samples, once the assay is complete, can lead to increased background and reduced sensitivity.

Table 2. Essential control assay tubes

Tube No.	Reagents (All reagent volumes are 50 µl)
1 (Negative Control 0 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Assay Diluent
2 (20 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Cytokine Standards 1:256 Dilution
3 (40 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Cytokine Standards 1:128 Dilution
4 (80 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Cytokine Standards 1:64 Dilution
5 (156 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Cytokine Standards 1:32 Dilution
6 (312 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Cytokine Standards 1:16 Dilution
7 (625 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Cytokine Standards 1:8 Dilution
8 (1250 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Cytokine Standards 1:4 Dilution
9 (2500 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Cytokine Standards 1:2 Dilution
10 (5000 pg/ml Standards)	mixed Capture Beads, PE Detection Reagent, Cytokine Standards "Top Standard"

Cytometer Setup, Data Acquisition and Analysis

The Cytometer setup information in this section is for the BD FACScan and BD FACSCalibur flow cytometers. The BD FACSComp™ software is useful for setting up the flow cytometer. BD CellQuest software is required for acquriring samples and formatting data for subsequent analysis using the BD CBA Software or FCAP Array Software.

Additional setup protocols for the BD FACSCalibur flow cytometer (dual laser), BD FACSArray™ bioanalyzer, and other BD FACS flow cytometers can be found at **bdbiosciences.com/cbasetup**

Preparation of 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 and protect from direct exposure to light.
5. Add 450 µl of Wash Buffer to tube A and 400 µl of Wash Buffer to tubes B and C.
6. Proceed to next section.

Instrument Setup with BD FACSComp Software and BD Calibrite Beads

1. Perform instrument start up.
2. Perform flow check.
3. Prepare tubes of BD Calibrite beads and open BD FACSComp software.
4. Launch BD FACSComp software
5. Run BD FACSComp software in Lyse/No Wash mode.
6. Proceed to next Section.

Note: For detailed information on using BD FACSComp with BD Calibrite beads to set up the flow cytometer, refer to the BD FACSComp Software User's Guide and the BD Calibrite Beads Package Insert. Version 4.2 contains a BD CBA preference setting to automatically save a BD CBA calibration file at the successful completion of any Lyse/No Wash assay. The BD CBA calibration file provides the optimization for FSC, SSC, and threshold settings as described in ***Instrument Setup with the Cytometer Setup Beads***, Steps 3 – 5. Optimization of the fluorescence parameter settings is still required (ie, PMT and compensation settings, see ***Instrument Setup with the Cytometer Setup Beads***, Step 6).

Instrument Setup with the Cytometer Setup Beads

1. Launch BD CellQuest Software and open the BD CBA Instrument Setup template.

   Note: The BD CBA Instrument Setup template can be found on the BD FACStation CD for Macintosh computers in the BD CBA folder. This file may also be downloaded from: **bdbiosciences.com/cbatemplates**

2. Set the instrument to Acquisition mode.

   Note: The data will be evaluated in five parameters (FSC, SSC, FL1, FL2 and FL3). Turn off additional detectors.

3. Set SSC (side light scatter) and FSC (forward light scatter) to Log mode.
4. Decrease the SSC PMT voltage by 100 from what BD FACSComp set.
5. Set the Threshold to SSC at 650.
6. In setup mode, run Cytometer Setup Beads tube A. Follow the setup instructions on pages 19 – 20.

   Note: Pause and restart acquisition frequently during the instrument setup procedure in order to reset detected values after settings adjustments.

Adjust gate R1 so that the singlet bead population is located in gate R1 (***Figure 3a***).

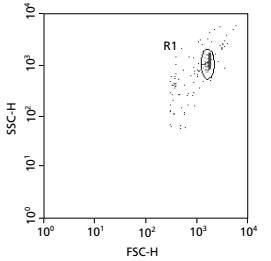


Figure 3a

Adjust the FL3 PMT so that the median intensity of the top FL3 bead population is around 5000 (***Figure 3b***). Adjust gate R3 as necessary so that the dim FL3 bead population is located in gate R3 (***Figure 3b***). Do not adjust the R2 gate.

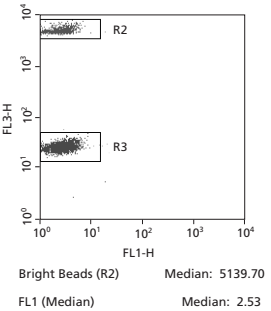


Figure 3b

Adjust the FL1 PMT so that the median of FL1 is approximately 2.0 – 2.5 (***Figure 3b***).

Adjust the FL2 PMT value so that the median of FL2 is approximately 2.0 – 2.5 (***Figure 3c***).

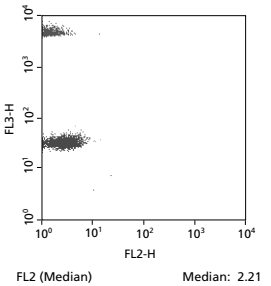


Figure 3c

Run Cytometer Setup Beads tube B to adjust the compensation settings for FL2 – %FL1.

Adjust gate R5 as necessary so that the FL1 bright bead population is located in gate R5 (***Figure 3d***). Using the FL2 – %FL1 control, adjust the median of R5 to equal the median of R4 (***Figure 3d***).

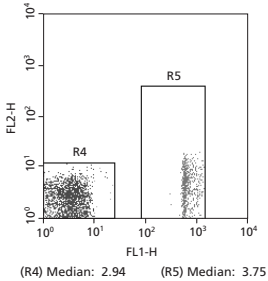


Figure 3d

Run Cytometer Setup Beads tube C to adjust the compensation settings for FL1 – %FL2 and FL3 – %FL2.

Adjust gate R7 so that the FL2 bright bead population is located in gate R7 (***Figure 3e***). Using the FL1 – %FL2 control, adjust the median of R7 to equal the median of R6 (***Figure 3e***).

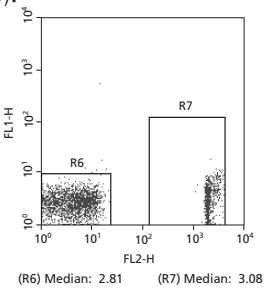


Figure 3e

Adjust gate R9 so that the FL2 bright bead population is located in gate R9 (***Figure 3f***). Using the FL3 – %FL2 control, adjust the median of R9 to equal the median of R8 (***Figure 3f***).

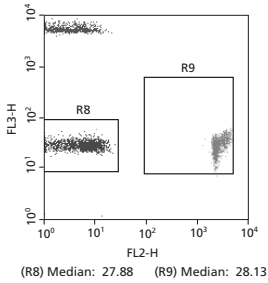


Figure 3f

Set the FL2 – %FL3 to 0.1 if necessary. Save and print the optimized instrument settings.

Data Acquisition

1. Open the acquisition template.

   Note: The acquisition template may be downloaded from: **bdbiosciences.com/cbatemplates**

2. Set acquisition mode and retrieve the optimized instrument settings from ***Instrument Setup with the Cytometer Setup Beads***, page 18.

3. In the Acquisition and Storage window, set the resolution to 1024.

4. Set number of events to be counted at 1800 of R1 gated events. (This will ensure that the sample file contains approximately 300 events per Capture Bead).

5. Set number of events to be collected to "all events". Saving all events collected will ensure that no true bead events are lost due to incorrect gating.

6. In setup mode, run tube no. 1 and using the FSC vs. SSC dot plot, place the R1 region gate around the singlet bead population *(*see ***Figure 3a)***.

7. Samples are now ready to be acquired.

8. Begin sample acquisition with the flow rate set at HIGH.

   Note: Run the negative control tube (0 pg/ml standards) before any of the recombinant standard tubes. Run the control assay tubes before any unknown test assay tubes. Run the tubes in the order listed in ***Table 2***, page 16.

   File names must be alphanumeric (ie, contain at least one letter).

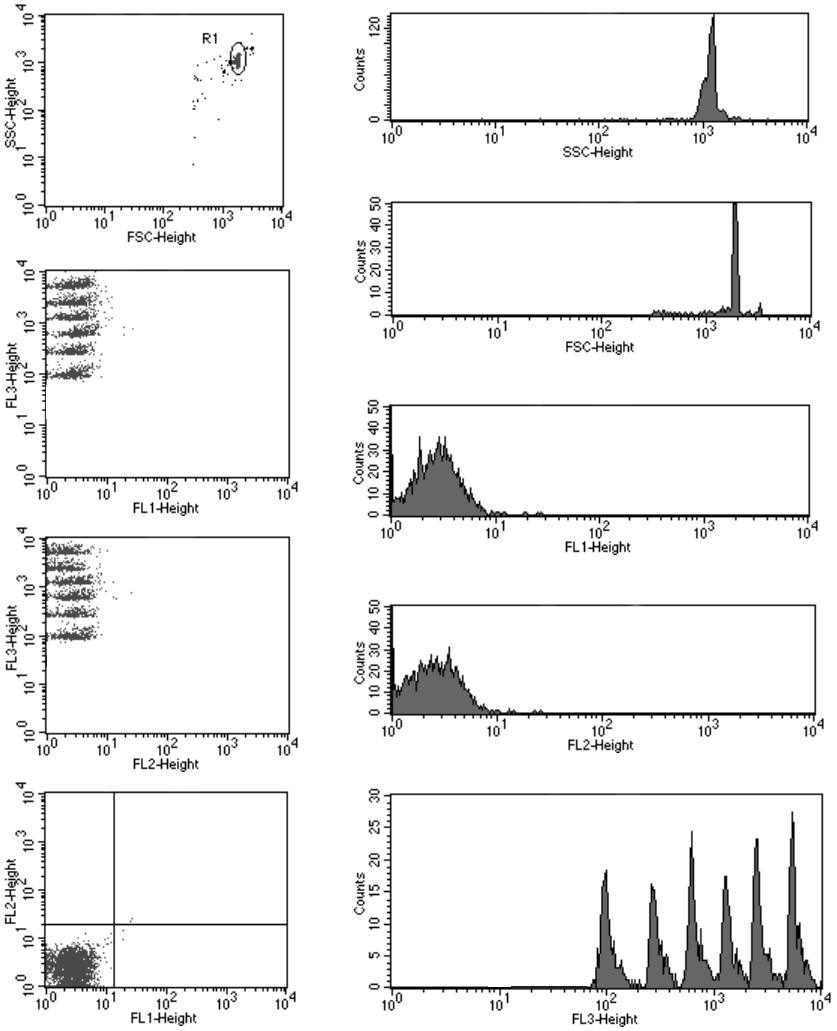


Figure 4. Acquisition Template Example

Analysis of Sample Data

The analysis of BD CBA data can be accomplished using BD CBA Software or FCAP Array Software. For BD CBA Software, please refer to the User's Guide for instructions. For FCAP Array Software, please visit the following link for instructions: **bdbiosciences.com/docs/FCAP_Array_analysis_of_CBA_Kits.pdf**

Typical Data

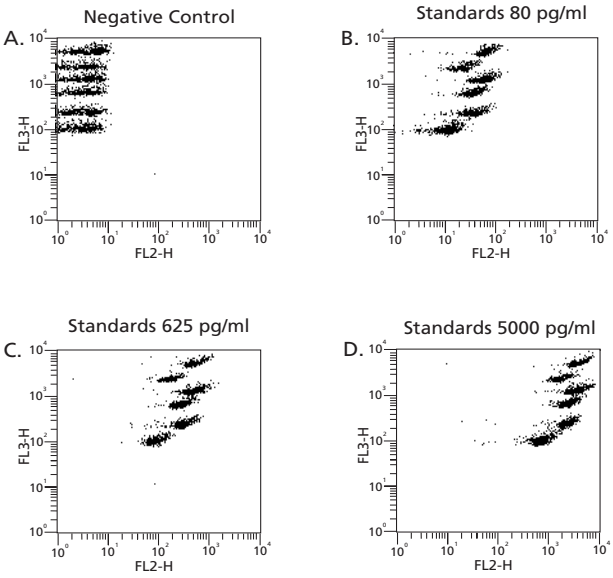


Figure 5. BD CellQuest Data Examples for Standards and Detectors Alone

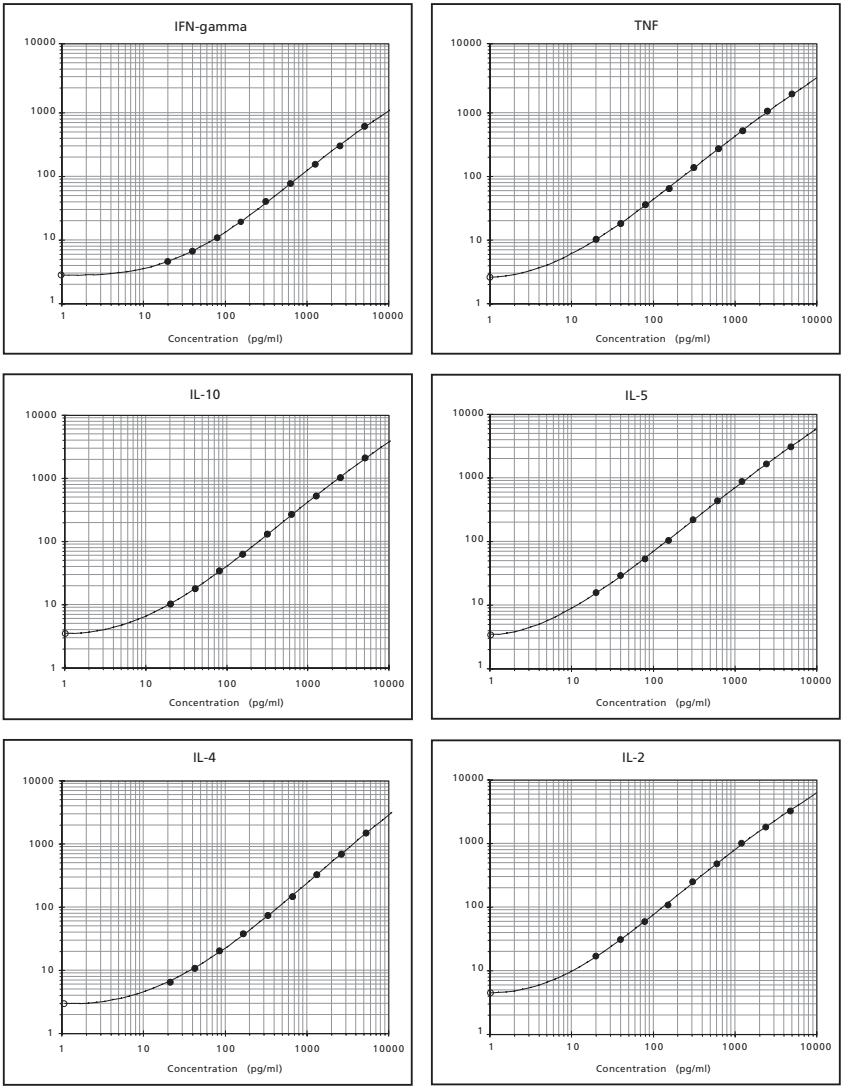


Figure 6. Example of Standard Curves

Performance

The BD CBA Human Th1/Th2 Cytokine Kit assay has been rigorously tested for the following: theoretical limit of detection, spike recovery, dilution linearity, specificity and intra- and inter-assay precision.

Theoretical Limit of Detection

The individual standard curve range for a given cytokine defines the minimum and maximum quantifiable levels using the BD CBA Human Th1/Th2 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 BD CBA Human 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).

Cytokine	Median Fluorescence	Standard Deviation	Limit of Detection (pg/ml)
IL-2	3.3	0.2	2.6
IL-4	2.3	0.2	2.6
IL-5	2.6	0.2	2.4
IL-10	2.4	0.2	2.8
TNF	2.0	0.2	2.8
IFN-γ	2.1	0.3	7.1

Recovery

Individual cytokine protein was spiked into various 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 Standard Diluent, as follows:

Cytokine	Matrix	Standard spike concentration (pg/ml)	Observed in given matrix (pg/ml)	% Recovery
IL-2	Pooled Donor Sera (*n* = 5)	2500	1958	78%
		625	406	65%
		80	49	62%
IL-2	Pooled Donor Plasma (*n* = 5)	2500	1656	78%
		625	427	68%
		80	54	67%
IL-2	Cell culture supernatant	2500	2605	104%
		625	659	106%
		80	86	107%
IL-4	Pooled Donor Sera (*n* = 5)	2500	2158	86%
		625	527	84%
		80	71	89%
IL-4	Pooled Donor Plasma (*n* = 5)	2500	2197	88%
		625	512	82%
		80	63	79%
IL-4	Cell culture supernatant	2500	2452	98%
		625	575	92%
		80	81	102%
IL-5	Pooled Donor Sera (*n* = 5)	2500	1748	70%
		625	430	69%
		80	58	72%
IL-5	Pooled Donor Plasma (*n* = 5)	2500	1710	68%
		625	380	61%
		80	54	67%
IL-5	Cell culture supernatant	2500	2622	105%
		625	625	102%
		80	85	106%
IL-10	Pooled Donor Sera (*n* = 5)	2500	2044	82%
		625	494	79%
		80	65	81%
IL-10	Pooled Donor Plasma (*n* = 5)	2500	2034	81%
		625	445	71%
		80	62	78%
IL-10	Cell culture supernatant	2500	2680	107%
		625	640	103%
		80	88	110%
TNF	Pooled Donor Sera (*n* = 5)	2500	1846	74%
		625	447	72%
		80	59	73%
TNF	Pooled Donor Plasma (*n* = 5)	2500	1731	69%
		625	407	65%
		80	54	67%
TNF	Cell culture supernatant	2500	2856	114%
		625	669	107%
		80	91	113%
IFN-γ	Pooled Donor Sera (*n* = 5)	2500	1748	70%
		625	479	77%
		80	73	91%
IFN-γ	Pooled Donor Plasma (*n* = 5)	2500	1813	73%
		625	458	73%
		80	66	83%
IFN-γ	Cell culture supernatant	2500	2446	98%
		625	628	101%
		80	84	105%

Linearity

In two experiments, the following matrices were spiked with IL-2, IL-4, IL-5, IL-10, TNF, and IFN-γ and were then serially diluted with Assay Diluent.

Matrix	Dilution	Observed IL-2 (pg/ml)	Observed IL-4 (pg/ml)	Observed IL-5 (pg/ml)
Pooled Donor Sera (*n* = 5)	Neat	3254	4079	3520
	1:2	1747	2292	2083
	1:4	863	1185	1150
	1:8	388	600	529
	1:16	187	277	275
	1:64	48	70	61
	1:256	12	19	17
	Slope	1.01	1.02	1.02
Pooled Donor Plasma (*n* = 5)	Neat	3785	4320	3750
	1:2	1934	2345	2047
	1:4	919	1181	1139
	1:8	433	608	541
	1:16	208	273	276
	1:64	53	67	68
	1:256	13	20	17
	Slope	1.01	1.01	1.01
Cell Culture Medium	Neat	4578	4546	4934
	1:2	2345	2409	2509
	1:4	1176	1105	1216
	1:8	561	605	614
	1:16	278	281	305
	1:64	70	77	72
	1:256	17	20	18
	Slope	1.01	1.01	1.00

Matrix	Dilution	Observed IL-10 (pg/ml)	Observed TNF (pg/ml)	Observed IFN-γ (pg/ml)
Pooled Donor Sera (*n* = 5)	Neat	3883	3502	3030
	1:2	2094	1971	1607
	1:4	1014	1054	892
	1:8	513	534	441
	1:16	244	270	227
	1:64	61	67	55
	1:256	17	18	20
	Slope	1.01	1.01	1.00
Pooled Donor Plasma (*n* = 5)	Neat	4245	3561	2743
	1:2	2118	2061	1580
	1:4	1032	1085	841
	1:8	532	554	407
	1:16	251	285	209
	1:64	68	75	50
	1:256	17	18	18
	Slope	1.00	1.01	1.02
Cell Culture Medium	Neat	4851	5500	4074
	1:2	2570	2762	2142
	1:4	1165	1222	1039
	1:8	610	613	506
	1:16	301	311	263
	1:64	80	78	57
	1:256	18	19	22
	Slope	1.01	1.00	1.01

Specificity

The antibody pairs used in the BD CBA Human Th1/Th2 Cytokine Kit assay 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.

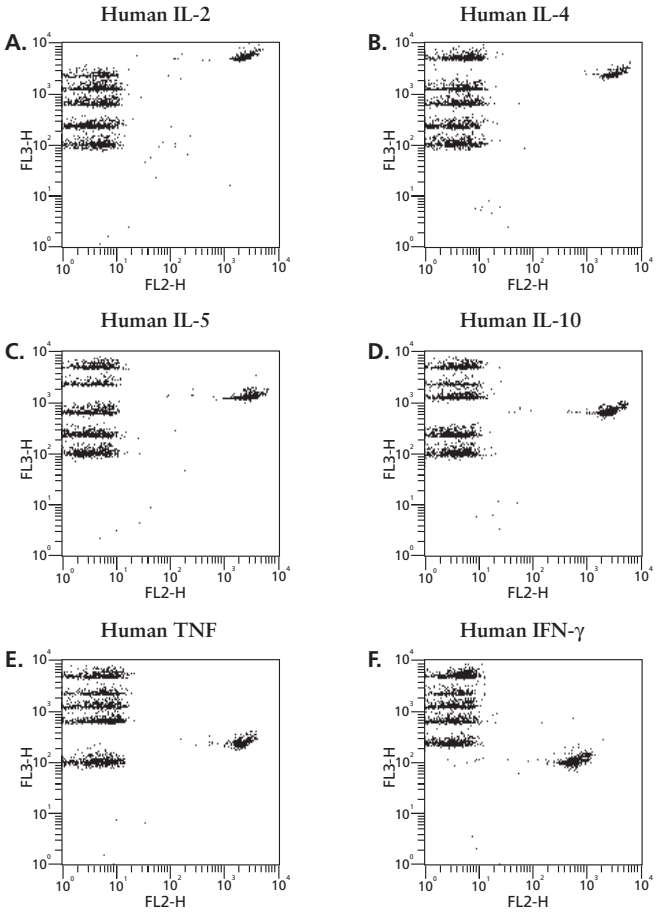


Figure 7. BD CellQuest Data for Detection of Individual Cytokines

Precision

Intra-assay: Ten replicates of each of three different levels of IL-2, IL-4, IL-5, IL-10, TNF, and IFN-γ were tested.

Cytokine	IL-2			IL-4		
Actual Mean Conc. (pg/ml):	71	534	2160	85	592	2488
SD	1	23	74	3	25	108
% CV	2%	4%	3%	4%	4%	4%

Cytokine	IL-5			IL-10		
Actual Mean Conc. (pg/ml):	78	587	2456	82	592	2486
SD	2	29	150	2	15	86
% CV	3%	5%	6%	2%	2%	3%

Cytokine	TNF			IFN-γ		
Actual Mean Conc. (pg/ml):	81	592	2504	78	528	2194
SD	2	22	126	3	21	65
% CV	3%	4%	5%	4%	4%	3%

Inter-assay: Three different levels of IL-2, IL-4, IL-5, IL-10, TNF, and IFN-γ (80, 625 and 2500 pg/ml) were tested in four experiments conducted by different operators.

Cytokine	IL-2			IL-4		
Number of Replicates:	8	8	8	8	8	8
Actual Mean Conc. (pg/ml):	77	627	2509	74	601	2553
SD	7	29	247	5	40	147
% CV	9%	5%	10%	7%	7%	6%

Cytokine	IL-5			IL-10		
Number of Replicates:	8	8	8	8	8	8
Actual Mean Conc. (pg/ml):	80	615	2560	81	631	2556
SD	4	39	165	5	45	189
% CV	5%	6%	6%	6%	7%	7%

Cytokine	TNF			IFN-γ		
Number of Replicates:	8	8	8	8	8	8
Actual Mean Conc. (pg/ml):	79	627	2564	69	610	2518
SD	4	31	131	6	40	139
% CV	5%	5%	5%	9%	7%	6%

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

Troubleshooting Tips

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 Human Th1/Th2 PE Detection Reagent by increasing the number of wash steps as 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 FSC threshold or further dilute samples. Increase 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.
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.

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

Note: The BD CBA Human Th1/Th2 Cytokine Kit assay has been shown to detect non-human primate IL-4, IL-5, TNF, and IFN-γ proteins produced by the activation of cells from rhesus and cynomolgus macaques. Direct quantitation of cytokines from non-human primates has not been validated using this kit and results may vary.

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Notes

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Notes

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