

Magnetic Luminex[®] Performance Assay

Human Cytokine Premixed Kit B

Catalog Number FCSTM04

For the simultaneous quantitative determination of multiple human cytokine concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Cytokines are intercellular signaling proteins released from a wide variety of cells and tissues. They play an integral role in regulating growth and cellular proliferation as well as modulating host response to infection, injury, and inflammation. Cytokines also influence reproduction and bone remodeling. A large number of cytokines are pleiotropic and share similar functions. In addition, many cytokines influence the production of other cytokines. Analysis and quantification of cytokines in biological fluids and cell culture supernates has thus become increasingly important. Methods such as bioassay, enzyme-linked immunosorbent assay (ELISA), intracellular staining, ribonuclease protection assay (RPA) and polymerase chain reaction (PCR) have all been used for quantifying cytokines; however, each of these techniques has limitations associated with it. These techniques are not capable of measuring multiple cytokines simultaneously in a limited sample volume.

This kit can be used to simultaneously assess the levels of multiple cytokines in a single sample. For ease of use, the microparticles and the biotinylated detection antibodies are premixed in respective vials.

Analyte	Performance Data Online (www.RnDSystems.com/pdf/...)	Microparticle Region
CCL11/Eotaxin	LUBM320.pdf	22
CD40 Ligand/TNFSF5	LUBM201.pdf	27
CXCL10/IP-10	LUBM266.pdf	38
CXCL11/I-TAC	LUBM672.pdf	42
EGF	LUBM236.pdf	51
HGF	LUBM294.pdf	53
IL-12 p70	LUBM219.pdf	43
IL-13	LUBM213.pdf	44
Leptin	LUBM398.pdf	45

PRINCIPLE OF THE ASSAY

Magnetic Luminex® Performance Assay multiplex kits are designed for use with the Luminex® MAGPIX® CCD Imager. Alternatively, kits can be used with the Luminex® 100/200™, Luminex® FLEXMAP 3D®, or Bio-Rad® Bio-Plex®, dual laser, flow-based sorting and detection platforms.

Analyte-specific antibodies are pre-coated onto magnetic microparticles embedded with fluorophores at set ratios for each unique microparticle region. Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the biotinylated antibody, is added to each well. Final washes remove unbound Streptavidin-PE, the microparticles are resuspended in buffer and read using the Luminex® MAGPIX® Analyzer. A magnet in the analyzer captures and holds the superparamagnetic microparticles in a monolayer. Two spectrally distinct Light Emitting Diodes (LEDs) illuminate the microparticles. One LED excites the dyes inside each microparticle to identify the region and the second LED excites the PE to measure the amount of analyte bound to the microparticle. A sample from each well is imaged with a CCD camera with a set of filter to differentiate excitation levels.

Analysis with the Luminex® 100/200™, Luminex® FLEXMAP 3D®, or Bio-Rad Bio-Plex uses one laser to excite the dyes inside each microparticle to identifying the microparticle region and the second laser to excite the PE to measure the amount of analyte bound to the microparticle. All excitation emitted as each microparticle passes through the flow cell is then analyzed to differentiate excitation levels using a Photomultiplier Tube (PMT) and an Avalanche Photodiode.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples fall outside the dynamic range of the assay, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until these factors have been tested in the Magnetic Luminex® Performance Assay, the possibility of interference cannot be excluded.
- Magnetic Luminex® Performance Assays afford the user the benefit of multianalyte analysis of biomarkers in a single complex sample. For each sample type, a single multipurpose diluent is used to optimize recovery, linearity, and reproducibility. Such a multipurpose diluent may not optimize any single analyte to the same degree that a unique diluent selected for analysis of that analyte can optimize conditions. Therefore, some performance characteristics may be more variable than those for assays designed specifically for single analyte analysis.
- **Only the analytes listed on the Standard Value Card can be measured with this kit. Refer to the enclosed certificate of analysis for specific analytes included in this kit.**

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED, DILUTED, OR RECONSTITUTED MATERIAL
Human Standard Cocktail 3	892794	2 vials of recombinant human cytokines in a buffered protein base with preservatives; lyophilized.	Discard after use. Use a fresh standard for each assay.
Human Cytokine Premixed B Magnetic Microparticle	898712	1.2 mL of a concentrated microparticle cocktail with preservatives.	May be stored for up to 1 month at 2-8 °C.* <i>Once diluted, 1X solutions must be discarded. Use fresh diluents for each assay.</i>
Human Cytokine Premixed B Biotin Antibody	894122	1.2 mL of a concentrated biotinylated antibody cocktail with preservatives.	
Streptavidin-PE Concentrate	892525	0.07 mL of a concentrated streptavidin-phycoerythrin conjugate with preservatives.	
Microparticle Diluent 4	895547	6 mL of a buffered protein base with preservative.	May be stored for up to 1 month at 2-8 °C.*
Biotin Antibody Diluent 2	895832	5.5 mL of a buffered protein base with preservative.	
Calibrator Diluent RD5-3	895436	21 mL of a buffered protein base with preservatives. <i>For cell culture supernate samples.</i>	
Calibrator Diluent RD6-43	895548	21 mL of a buffered protein base with preservatives. <i>For serum/plasma samples.</i>	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. <i>May turn yellow over time.</i>	
Microplate	641385	1 flat-bottomed 96-well microplate used as a vessel for the assay.	
Mixing Bottles	895505	2 empty 8 mL bottles used for mixing microparticles with Microparticle Diluent.	
Plate Sealers	640445	4 adhesive foil strips.	
Standard Value Card	750636	1 card listing the Standard Cocktail 3 reconstitution volume and working standard concentrations for this kit lot.	

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Luminex® MAGPIX®, Luminex® 100/200TM, Luminex® FLEXMAP 3D®, or Bio-Rad Bio-Plex analyzer with X-Y platform.
- Hand-held microplate magnet or platewasher with a magnetic platform.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Multi-channel pipette, manifold dispenser, or automated dispensing unit.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 800 ± 50 rpm.
- Microcentrifuge.
- **Polypropylene** test tubes for dilution of standards and samples.
- Luminex® Performance Assay Controls (optional; R&D Systems®, Catalog # QC10).

PRECAUTIONS

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Protect microparticles and Streptavidin-PE from light at all times to prevent photobleaching.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

Serum and EDTA plasma are not suitable for use in the IP-10 assay.

SAMPLE PREPARATION

Use polypropylene tubes.

Note: *On the day of the assay, ALL fresh and previously frozen serum and plasma samples require centrifugation at 16,000 x g for 4 minutes immediately prior to use or dilution.*

Serum and plasma samples require a 4-fold dilution. A suggested 4-fold dilution is 30 μL of sample + 90 μL of Calibrator Diluent RD6-43. Mix thoroughly.

When assaying IL-12 p70, cell culture supernate samples must be diluted 2-fold. A suggested 2-fold dilution is 75 μL of sample + 75 μL of Calibrator Diluent RD5-3. Mix thoroughly.

When assaying IP-10, cell culture supernate samples must be diluted 15-fold. A suggested 15-fold dilution is 10 μL of sample + 140 μL of Calibrator Diluent RD5-3. Mix thoroughly.

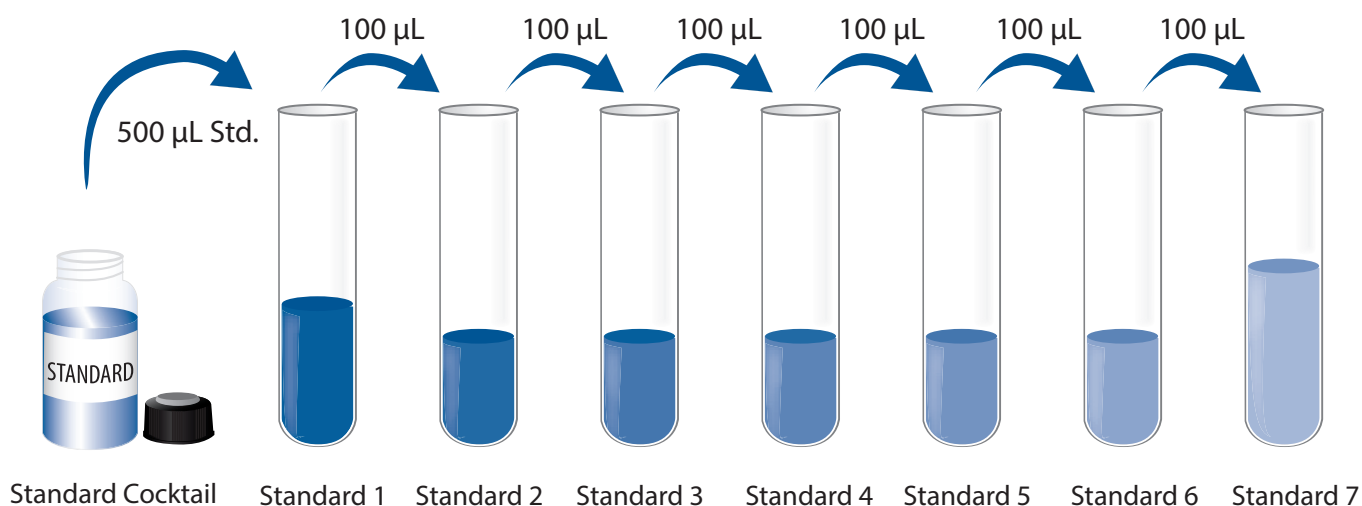
REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Standards - Refer to the Standard Value Card for the reconstitution volume and the assigned values. Reconstitute the Human Standard Cocktail 3 with Calibrator Diluent RD5-3 (*for cell culture supernate samples*) or Calibrator Diluent RD6-43 (*for serum/plasma samples*). Allow the standards to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 500 μ L of reconstituted Standard into a tube labeled working Standard 1. Pipette 200 μ L of the appropriate calibrator diluent into the remaining tubes. Use working Standard 1 to produce a 3-fold dilution series (below). Mix each tube thoroughly before the next transfer. Working Standard 1 serves as the high standard. The appropriate calibrator diluent serves as the blank.



DILUTED MICROPARTICLE COCKTAIL PREPARATION

1. Centrifuge the Microparticle Cocktail vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial to resuspend the microparticles, taking precautions not to invert the vial.
3. Dilute the Microparticle Cocktail in the mixing bottle provided.

Number of Wells Used	Microparticle Cocktail	+	Microparticle Diluent 4
96	1000 µL	+	4.5 mL
72	750 µL	+	3.375 mL
48	500 µL	+	2.25 mL
24	250 µL	+	1.125 mL

Note: Protect microparticles from light during handling. Diluted microparticles cannot be stored. Prepare microparticles within 30 minutes of use.

DILUTED BIOTIN-ANTIBODY COCKTAIL PREPARATION

1. Centrifuge the Biotin-Antibody Cocktail vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Dilute the Biotin-Antibody Cocktail in Biotin Antibody Diluent 2. Mix gently.

Number of Wells Used	Biotin-Antibody Cocktail	+	Biotin Antibody Diluent 2
96	1000 µL	+	4.5 mL
72	750 µL	+	3.375 mL
48	500 µL	+	2.25 mL
24	250 µL	+	1.125 mL

STREPTAVIDIN-PE PREPARATION

Use a polypropylene amber bottle or a polypropylene tube wrapped with aluminum foil. Protect Streptavidin-PE from light during handling and storage.

1. Centrifuge the Streptavidin-PE Concentrate vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Dilute the Streptavidin-PE concentrate in Wash Buffer.

Number of Wells Used	Streptavidin-PE Concentrate	+	Wash Buffer
96	55.0 µL	+	5.50 mL
72	42.0 µL	+	4.10 mL
48	28.0 µL	+	2.75 mL
24	14.0 µL	+	1.35 mL

INSTRUMENT SETTINGS

Note: *Adjust the probe height setting on the analyzer to avoid puncturing the plate. Calibrate the analyzer using the proper reagents for superparamagnetic microparticles (refer to instrument manual).*

Luminex® MAGPIX® analyzer:

- a) Sample volume: 50 µL
- b) Assign the microparticle region for each analyte being measured (see page 1)
- c) 50 count/region
- d) Collect Median Fluorescence Intensity (MFI)

Luminex® 100/200™, Luminex® FLEXMAP 3D® and Bio-Rad Bio-Plex analyzers:

Note: *Ensure that the instrument flow rate is set to the default of 60 µL/minute (fast) for all flow based analyzers.*

- a) Sample volume: 50 µL
- b) Bead Type:
 - i. Luminex® 100/200™ and FLEXMAP 3D® select MagPlex
 - ii. Bio-Rad Bio-Plex Manager use Bio-Plex MagPlex Beads (Magnetic)
- c) Doublet Discriminator gates:
 - i. Luminex® 100/200™ and FLEXMAP 3D® set at 8000 and 16,500
 - ii. Bio-Rad Bio-Plex Manager set at 8000 and 23,000
- d) Reporter Gain Setting:
 - i. Luminex® 100/200™ use Default setting
 - ii. Luminex® FLEXMAP 3D® use Standard PMT setting
 - iii. Bio-Rad Bio-Plex Manager use the low RP1 target value for the CAL2 setting
- e) Assign the microparticle region for each analyte being measured (see page 1)
- f) 50 count/region
- g) Collect MFI

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

Note: *Protect microparticles and Streptavidin-PE from light at all times.*

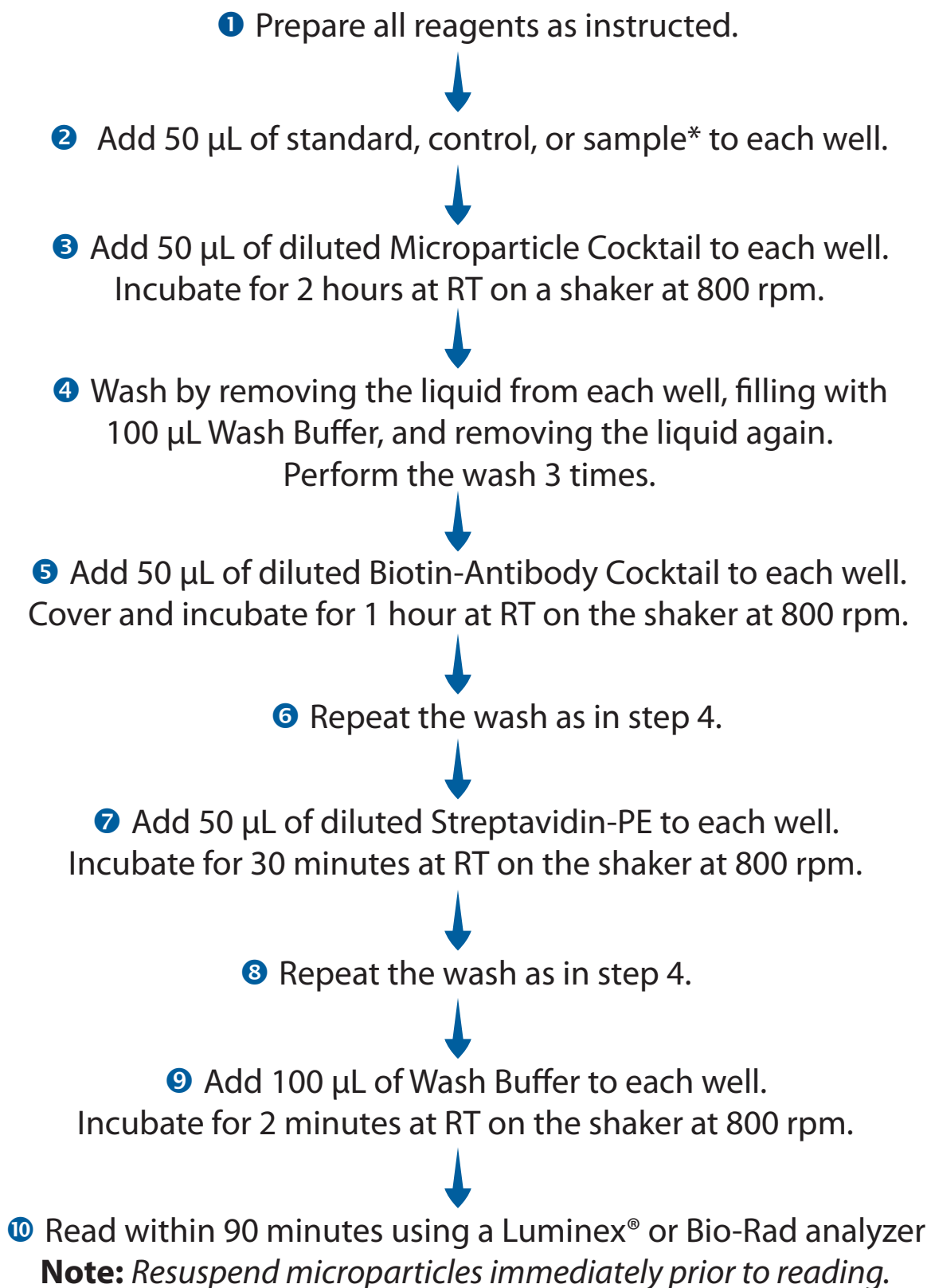
1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Add 50 μ L of standard, control, or sample* per well. A plate layout is provided to record standards and samples assayed.
3. Resuspend the diluted Microparticle Cocktail by inversion or vortexing. Add 50 μ L of the Microparticle Cocktail to each well of the microplate. Securely cover with a foil plate sealer. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 800 ± 50 rpm.
4. Using a magnetic device designed to accommodate a microplate, wash by applying the magnet to the bottom of the microplate, allow 1 minute before removing the liquid, filling each well with Wash Buffer (100 μ L) and allow 1 minute before removing the liquid again. Complete removal of liquid is essential for good performance. **Note: Do NOT blot; this may cause a loss of microparticles.** Perform the wash procedure three times.

Note: *Refer to the magnetic device user manual for proper wash technique using a round bottom microplate.*
5. Add 50 μ L of diluted Biotin-Antibody Cocktail to each well. Securely cover with a foil plate sealer and incubate for 1 hour at room temperature on the shaker set at 800 ± 50 rpm.
6. Repeat the wash as in step 4.
7. Add 50 μ L of diluted Streptavidin-PE to each well. Securely cover with a foil plate sealer and incubate for 30 minutes at room temperature on the shaker set at 800 ± 50 rpm.
8. Repeat the wash as in step 4.
9. Resuspend the microparticles by adding 100 μ L of Wash Buffer to each well. Incubate for 2 minutes on the shaker set at 800 ± 50 rpm.
10. Read within 90 minutes using the Luminex® or Bio-Rad analyzer.
Note: *Resuspend microparticles immediately prior to reading by shaking the plate for 2 minutes on the plate shaker set at 800 ± 50 rpm.*

*Samples may require dilution. See Sample Preparation section.

ASSAY PROCEDURE SUMMARY

Note: Protect microparticles and Streptavidin-PE from light at all times.



*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Use the Standard concentrations on the Standard Value Card and calculate 3-fold dilutions for the remaining levels. Average the duplicate readings for each standard and sample and subtract the average blank Median Fluorescence Intensity (MFI).

Create a standard curve for each analyte by reducing the data using computer software capable of generating a five parameter logistic (5-PL) curve-fit.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

CALIBRATION

This assay is calibrated against highly purified recombinant human cytokines produced at R&D Systems®.

SPECIFICITY

The assay was tested for cross-reactivity and interference with the following factors. Less than 0.5% cross-reactivity and interference was observed unless otherwise noted on the analyte specific datasheet.

Recombinant human:

6Ckine	IL-5
Angiogenin	IL-6
Ang-2	IL-7
BDNF	IL-8
BMP-2	IL-9
BMP-4	IL-10
BMP-5	IL-11
BMP-6	IL-12/IL-35 p35
CD4	IL-12/IL-23 p40
CD40	IL-15
CNTF	IL-16
CT-1	IL-17
CTLA-4	LIF
ENA-78	MCP-1
Eotaxin-2	MCP-2
Eotaxin-3	MCP-3
Eotaxin-3 (24-94 aa)	MIP-1 α
Epo	MIP-1 α (70 aa)
Fas	MIP-1 β
FGF basic	MIP-1 δ (9 aa)
Follistatin ₂₈₈	MIP-1 δ (68 aa)
Follistatin ₃₀₀	MIPF-1
Follistatin ₃₁₅	RANTES
G-CSF	TGF- β 1
GDNF	TGF- β 1.2
GITR	TGF- β 2
GM-CSF	TGF- β 3
IFN- γ	TGF- β RII
IL-1 α	TGF- β RIII
IL-1 β	TNF- α
IL-1ra	TNF- β
IL-2	Tpo
IL-3	TRAIL
IL-4	TRANCE

Recombinant rat:

CRP
Pentraxin 2/SAP
TIMP-1

Recombinant porcine:

MIP-3 α

Recombinant human multiplex partners:

CD40 Ligand
EGF
Eotaxin
HGF
IL-12 p70
IL-13
IP-10
I-TAC
Leptin

PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H

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