Non-Human Primate Cytokine Magnetic Bead Panel

96 Well Plate Assay

Cat. # PRCYTOMAG-40K
Cat. # PCYTMG-40K-PX23

MILLIPLEX® MAP

NON-HUMAN PRIMATE CYTOKINE MAGNETIC BEAD PANEL KIT
96 Well Plate Assay

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FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES

Introduction

"Cytokine" is a general term used for a diverse group of soluble proteins and peptides which act as regulators under both normal and pathological conditions to modulate the functional activities of individual cells and tissues. These proteins also mediate interactions between cells directly and regulate processes taking place in the extracellular environment. Cytokines differ from hormones in that they act on a wider spectrum of target cells and also they are not produced by specialized cells which are organized in specialized glands. This group of proteins includes lymphokines, interferons, colony stimulating factors and chemokines.

Cytokine and chemokine research plays a significant role in achieving a deeper understanding of disease states such as allergic reactions, IBD, sepsis, and cancer. Therefore, the MILLIPLEX MAP Non-Human Primate Cytokine Panel enables you to focus on the therapeutic potential of cytokines as well as the modulation of cytokine expression. Coupled with the Luminex xMAP® platform, you receive the advantage of ideal speed and sensitivity, allowing quantitative multiplex detection of dozens of analytes simultaneously which can dramatically improve productivity.

Using Luminex xMAP technology, EMD Millipore has developed the MILLIPLEX MAP Non-Human Primate Cytokine Multiplex Panel. Magnetic Beads can make the process of automation and high throughput screening easier with features such as walk-away washing. Advantages even outside automation include:

- More flexible plate and plate washer options.
- Improved performance with turbid serum/plasma samples.
- Assay results equivalent to non-magnetic beads.
- Automated washing eliminates technical obstacles (e.g., clogging of wells that contain viscous samples) which may result during vacuum manifold/manual washing.

EMD Millipore's MILLIPLEX MAP Non-Human Primate Cytokine panel is the most versatile system available for Cytokine research.

- MILLIPLEX MAP offers you the ability to:
    - Select a 23-plex premixed kit or
    - Choose any combination of analytes from our panel of 23 analytes to design a custom kit that better meets your needs.
- A convenient "all-in-one" box format gives you the assurance that you will have all the necessary reagents you need to run your assay.

EMD Millipore's MILLIPLEX MAP Non-Human Primate Cytokine panel kit is to be used for the

simultaneous quantification of G-CSF, GM-CSF, IFNγ, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12/23(p40), IL-13, IL-15, IL-17A, MCP-1, MIP-1β, MIP-1α, sD40L, TGF-α, TNF-α, VEGF, and IL-18. This kit may be used for the analysis of all or any combination of the above analytes in tissue/cell lysate and culture supernatant samples and serum or plasma samples

This kit is for research purposes only.

Please read entire protocol before use.

It is important to use same assay incubation conditions throughout your study.

Alternative Names of Cytokines/Chemokines

Cytokine	Name	Alternative Name
G-CSF	Granulocyte colony stimulating factor	DF, GM-DF, MGI-2, NAP-IF, Pluriopoietin, PCSF, 5637-derived factor, Pluripoietin-beta
GM-CSF	Granulocyte-macrophage colony stimulating factor	CSF-alpha, CSF-beta, CSF-2, FDCP1 growth factor, GM-CSA, HCSF, KM102-BPA, KTGF, LBGF, NIF-T, Pluripoietin- alpha, TPO, TSF, burst promoting activity (BPA)
IFNγ	Gamma Interferon	Antigen induced Interferon, immune interferon, type 2 interferon, T-interferon, Mitogen induced interferon, pH2-labile interferon
IL-1β	Interleukin 1 Beta	Catabolin, H1, IFN-beta inducing factor, Interleukin -beta, OAF,
IL-1ra	IL-1 receptor antagonist	IL1RN; IL1RA; ICIL-1RA; IL-1ra3; IL1F3; IRAP; MGC10430
IL-2	Interleukin 2	BF, Costimulator, LMF, LCM factor, LPF, MAF-C1, PFC-EA, SCIF, TCGF, TCPA, TDF, T-LPF, TMF, TSF
IL-4	Interleukin 4	IaIF , BSF-1, BCDF-gamma, BCGF-gamma , BCGF-1, BSF-1, BSF-p1 , EL4-BCGF, IgE-EF, IgE switch factor, IgG1-enhancing factor, IgG1-induction factor, LMW-BCGF, MCGF-2 , TCGF-2
IL-5	Interleukin 5	B151-TRF , BCDF-mu , BCGF-2, DL-BCGF, CFU-Eo GSF, EDF, Eo-CSF, Eo-DF, IgA-EF, TRF-1
IL-6	Interleukin 6	26 kDa protein, BSF-2 ,BSF-p2 , CSF-309 , DIF, FDGI , HGI, HSF, HSF-1,IFN-beta-2, ILHP1 , MGI-2A , Myeloma GF , Natural killer cell activity-augmenting factor , WI-26-VA4 factor , CPA
IL-8	Interleukin 8	3-10C; AMCF-I; CXCL8; GCP-1; GCP1; K60; LECT; LUCT; LYNAP; MDNCF; MONAP; NAF; NAP-1; NAP1; SCYB8; TSG-1; b-ENAP
IL-10	Interleukin 10	B-TCGF, CSIF, TGIF
IL-12/23(p40)	Interleukin 12, heterodimeric glycoprotein	CLMF, NKSF, TSF
IL-13	Interleukin 13	NC30
IL-15	Interleukin 15	IL-T
IL-17A	Interleukin 17	IL-17A
MCP-1	Monocyte chemoattractant protein-1	SCYA2, CCL2, GDCF, HC11, LDCF, MCAF, MCP, SMC-CF, TDCF,TSG-8
MIP-1α	Macrophage inflammatory protein-1-alpha	CCL3
MIP-1β	Macrophage inflammatory protein-1-beta	CCL4, ACT-2, LAG-1
sCD40L	soluble CD40 ligand	TRAP, CD154
TGFα	Transforming growth factor alpha	ETGF, TFGA
TNFα	Tumor necrosis factor-alpha	Cachectin, cytotoxic factor (CF), CTX, Hemorrhagic factor, Macrophage-derived cytotoxic factor, macrophage cytotoxic factor (MCF), MCT, TNFSF2
VEGF	Vascular endothelial growth factor	Mouse sarcoma 180-derived growth factor, Vasculotropin.(VAS), Vascular endothelial cell proliferation factor , VPF, GD-VEGF, VEGF-A
IL-18	Interleukin 18	IGIF; IL-1g; IL1F4; MGC12320

PRINCIPLE

MILLIPLEX MAP are based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life-sciences and capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded magnetic beads known as MagPlex™-C microspheres.

- Luminex® uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, 100 distinctly colored bead sets can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- The microspheres are allowed to pass rapidly through a laser which excites the internal dyes marking the microsphere set. A second laser excites PE, the fluorescent dye on the reporter molecule.
- Finally, high-speed digital-signal processors identify each individual microsphere and quantify the result of its bioassay based on fluorescent reporter signals.

The capability of adding multiple conjugated beads to each sample results in the ability to obtain multiple results from each sample. Open-architecture xMAP® technology enables multiplexing of many types of bioassays reducing time, labor and costs over traditional methods.

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze/ thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
Non-Human Primate Cytokine Standard	MXPR8040	lyophilized	1 vial
Non-Human Primate Cytokine Quality Controls 1 and 2	MXPR6040	lyophilized	2 vials
Serum Matrix (for serum/plasma samples only)	MXPRSM	lyophilized	1 vial
Set of one 96-Well Plate with 2 sealers	----------	----------	1 plates 2 sealers
Assay Buffer Note: Contains 0.05% Proclin	L-AB	30 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	2 bottle
Non-Human Primate Cytokine Detection Antibodies	MXPR1040-2	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE4	3.2 mL	1 bottle
Bead Diluent (not provided with premixed panel)	LBD	3.5 mL	1 bottle
Mixing Bottle (not provided with premixed panel)	----------	----------	1 bottle

Non-Human Primate Cytokine Antibody-Immobilized Premixed Beads:

Premixed 23-plex Beads	PRCYPMX23-MAG	3.5 mL	1 bottle

Included Non-Human Primate Cytokine Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel (see following table page 7).

Non-Human Primate Cytokine Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex magnetic Bead Region	Customizable 23 Analytes (50X Concentration, 90 µL)		23-Plex Premixed Beads
		Available	Cat. #	
Anti- G-CSF Bead	18	□	HGCSF-MAG	□
Anti- GM-CSF Bead	14	□	PRGMCSF-MAG	□
Anti- IFNγ Bead	20	□	PRIFNG-MAG	□
Anti- IL-1β Bead	46	□	HCYIL1B-MAG	□
Anti- IL-1ra Bead	42	□	HIL1RA-MAG	□
Anti- IL-2 Bead	33	□	PRIL2-MAG	□
Anti- IL-4 Bead	53	□	HIL4-MAG	□
Anti- IL-5 Bead	55	□	HIL5-MAG	□
Anti- IL-6 Bead	57	□	HCYIL6-MAG	□
Anti- IL-8 Bead	63	□	HCYIL8-MAG	□
Anti- IL-10 Bead	35	□	PRIL10-MAG	□
Anti- IL-12/23(p40) Bead	74	□	PRIL12P40-MAG	□
Anti- IL-13 Bead	44	□	PRIL13-MAG	□
Anti- IL-15 Bead	37	□	HIL15-MAG	□
Anti- IL-17A Bead	39	□	HIL17-MAG	□
Anti-IL-18 Bead	78	□	PRIL18-MAG	□
Anti- MCP-1 Bead	67	□	HCYMCP1-MAG	□
Anti- MIP-1β Bead	73	□	HMIP1B-MAG	□
Anti- MIP-1α Bead	65	□	PRMIP1A-MAG	□
Anti- sCD40L Bead	38	□	HCD40L-MAG	□
Anti-TGFα Bead	15	□	HCYTGFA-MAG	□
Anti- TNFα Bead	72	□	PRTNFA-MAG	□
Anti- VEGF Bead	76	□	PRVEGF-MAG	□

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (Luminex Catalogue #40-50000) or Luminex Drive Fluid (Luminex Catalogue # MPXDF-4PK)

Instrumentation / Materials

1. Adjustable Pipettes with Tips capable of delivering 25 µL to 1000 µL
2. Multichannel Pipettes capable of delivering 5 µL to 50 µL or 25 µL to 200 µL
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. Rubber Bands
6. Aluminum Foil
7. Absorbent Pads
8. Laboratory Vortex Mixer
9. Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
10. Titer Plate Shaker (Lab-Line Instruments Model #4625 or equivalent)
11. Luminex 200™, HTS, FLEXMAP 3D™, or MAGPIX® with xPONENT software by Luminex Corporation
12. Automatic Plate washer for magnetic beads (Bio-Tek ELx405, EMD Millipore catalog #40-015 or equivalent) or Hand held Magnetic Separation Block (EMD Millipore catalog # 40-285 or equivalent)

Note: If a plate washer or hand held magnetic separation block for magnetic beads is not available, one can use a microtiter filter plate (EMD Millipore Catalog #MX-PLATE) to run the assay using a Vacuum Filtration Unit (EMD Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with EMD Millipore Vacuum Pump Catalog #WP6111560 or equivalent).

SAFETY PRECAUTIONS

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, sodium azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. On disposal, flush with a large volume of water to prevent azide build up.

TECHNICAL GUIDELINES

To obtain reliable and reproducible results, the operator should carefully read this entire manual and fully understand all aspects of each assay step before running the assay. The following notes should be reviewed and understood before the assay is set up.

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing the beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at $\leq$ -20°C for 1 month and at $\leq$ -80°C for greater than one month.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8°C for up to one month.
- During the preparation of the standard curve, make certain to mix the higher concentration well before making the next dilution. Use a new tip with each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some analytes.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.

TECHNICAL GUIDELINES (continued

- When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 4 alignment discs. When reading the assay on FLEXMAP 3D™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc. When reading the assay on MAGPIX, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 2 alignment disc.
- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells. If samples are diluted in assay buffer, use the assay buffer as matrix.
- For serum/plasma samples that require further dilution use the Serum Matrix provided in the kit for further dilution.
- For cell/tissue homogenate, the final cell or tissue homogenate should be prepared in a buffer that has a neutral pH, contains minimal detergents or strong denaturing detergents, and has an ionic strength close to physiological concentration. Avoid debris, lipids, and cell/tissue aggregates. Centrifuge samples before use.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000xg. Remove serum and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Customers should determine the optimal dilution for their samples. Generally, serum or plasma samples from normal subjects do not need dilution and can be tested directly. However, for those samples that are out of standard range, serum or plasma samples should be diluted using the reconstituted Serum Matrix as the sample diluent.

B. Preparation of Plasma Samples:

- Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1000xg within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.

- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Customers should determine the optimal dilution for their samples. Generally, serum or plasma samples from normal subjects do not need dilution and can be tested directly. However, for those samples that are out of standard range, serum or plasma samples should be diluted using the reconstituted Serum Matrix as the sample diluent.

C. Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- Tissue culture supernatant may require a dilution with an appropriate control medium prior to assay. Tissue/cell extracts should be done in neutral buffers containing reagents and conditions that do not interfere with assay performance. Excess concentrations of detergent, salt, denaturants, high or low pH, etc. will negatively affect the assay. Organic solvents should be avoided. The tissue/cell extract samples should be free of particles such as cells or tissue debris.

NOTE:

- A maximum of 25 µL per well of serum or plasma can be used. Tissue culture or other media may also be used.
- All samples must be stored in polypropylene tubes. **DO NOT STORE SAMPLES IN GLASS.**
- Avoid debris, lipids and cells when using samples with gross hemolysis or lipemia.
- Care must be taken when using heparin as an anticoagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

If premixed beads are used, sonicate the premixed bead vial 30 seconds and then vortex for 1 minute before use. (Note: Due to the composition of magnetic beads, you may notice a slight color in the bead solution. This does not affect the performance of the beads or the kit.)

For individual vials of beads, sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 60 µL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent. Vortex the mixed beads well. Unused portions may be stored at 2-8°C for up to one month. (Note: Due to the composition of magnetic beads, you may notice a slight color in the bead solution. This does not affect the performance of the beads or the kit.)

Example 1: When using 10 cytokine antibody-immobilized beads, add 60 µL from each of the 10 bead sets to the Mixing Bottle. Then add 2.4 mL Bead Diluent.

Example 2: When using 5 cytokine antibody-immobilized beads, add 60 µL from each of the 5 bead sets to the Mixing Bottle. Then add 2.7 mL Bead Diluent.

B. Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 µL deionized water. Invert the vial several times to mix and vortex. Allow the vial to sit for 5-10 minutes. Unused portion may be stored at ≤ -20°C for up to one month.

C. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 60 mL (2 bottles) of 10X Wash Buffer with 540 mL deionized water. Store unused portion at 2-8°C for up to one month.

D. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Add 1.0 mL deionized water to the bottle containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution. Leftover reconstituted Serum Matrix should be stored at ≤ -20°C for up to one month.

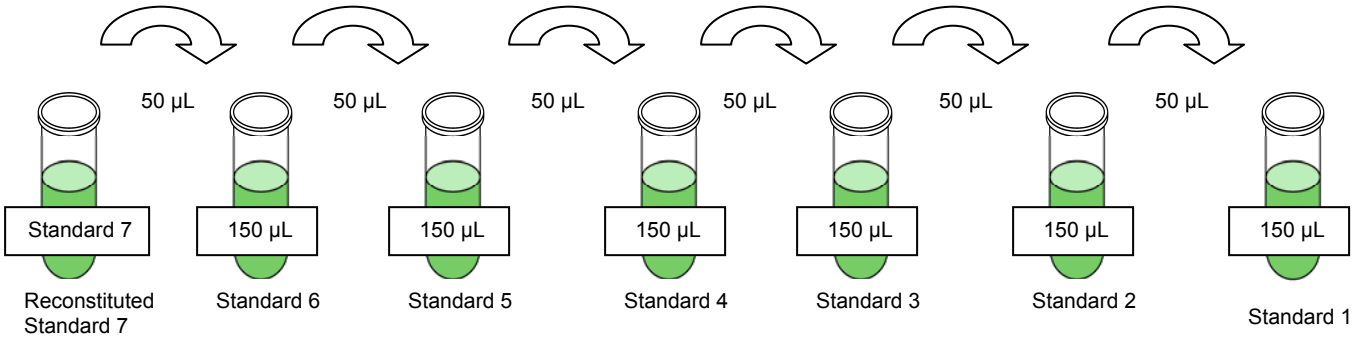
E. Preparation of Non-Human Primate Cytokine Panel Standard

1) Prior to use, reconstitute the Non-Human Primate Cytokine Panel Standard with 250 µL deionized water. Invert the vial several times to mix and vortex for 5-10 seconds. Allow the vial to sit for 5-10 minutes mix well. This will be Standard 7.

2) Label six polypropylene microfuge tubes Standard 6, Standard 5, Standard 4, Standard 3, Standard 2, and Standard 1, and add 150 µL of Assay Buffer to each of the six tubes. Perform 4-fold serial dilutions by adding 50 µL of the Standard 7 to the Standard 6 tube, mix well and transfer 50 µL of the Standard 6 to the Standard 5 tube, mix well and transfer 50 µL of the Standard 5 to the Standard 4 tube, mix well and transfer 50 µL of the Standard 4 to the Standard 3 tube, mix well and transfer 50 µL of the Standard 3 to the Standard 2 tube, mix well and transfer 50 µL of the Standard 2 to the Standard 1 tube, and mix Standard 1 well. The Standard 0 (Background) will be Assay Buffer.

Standard Tube #	Volume of Deionized Water to Add	Volume of Standard to Add
Standard 7 (reconstituted standard)	250 µL	0

Standard Tube #	Volume of Assay Buffer to Add	Volume of Standard to Add
Standard 6	150 µL	50 µL of Standard 7
Standard 5	150 µL	50 µL of Standard 6
Standard 4	150 µL	50 µL of Standard 5
Standard 3	150 µL	50 µL of Standard 4
Standard 2	150 µL	50 µL of Standard 3
Standard 1	150 µL	50 µL of Standard 2



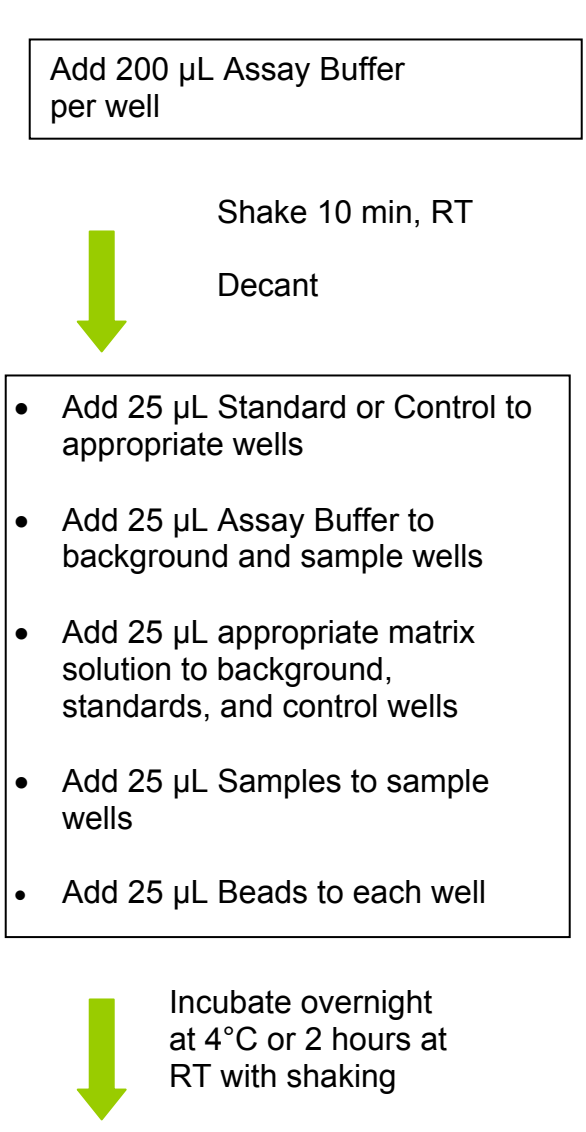
After serial dilutions, the tubes should have the following concentrations for constructing standard curves.

Standard Tube #	IL-4 (pg/mL)	IL-10, IL-18 (pg/mL)	All Other Analytes (pg/mL)
1	4.9	12.2	2.4
2	19.5	48.8	9.8
3	78.1	195.3	39
4	312.5	781.3	156.3
5	1,250	3,125	625
6	5,000	12,500	2,500
7	20,000	50,000	10,000

IMMUNOASSAY PROCEDURE

- Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines.
- Allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Diagram the placement of Standards [0 (Background), 1, 2, 3, 4, 5, 6, 7], Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will only read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.
- If using a filter plate, set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Add 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for Standard 0 (Background).
4. Add 25 µL of Assay Buffer to the sample wells.
5. Add 25 µL of appropriate matrix solution to the background, standards, and control wells. When assaying serum or plasma, use the Serum Matrix provided in the kit. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
6. Add 25 µL of Sample into the appropriate wells.
7. Vortex Mixing Bottle and add 25 µL of the Mixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-18 hr) at 4°C or 2 hours at room temperature (20-25° C). *An overnight incubation may improve assay sensitivity for some analytes.*



9. Gently remove well contents and wash plate 2 times following instructions listed in the **PLATE WASHING** section.
10. Add 25 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)
11. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT ASPIRATE AFTER INCUBATION.**
12. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.
13. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
14. Gently remove well contents and wash plate 2 times following instructions listed in the **PLATE WASHING** section.
15. Add 150 µL of Sheath Fluid (or Drive Fluid is using MAGPIX) to all wells. Resuspend the beads on a plate shaker for 5 minutes.
16. Run plate on Luminex 200 , HTS or FLEXMAP 3D or MAGPIX® with xPONENT software.
17. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. (Note: If samples are diluted, multiply the result by the dilution factor.)

Remove well contents and wash 2X with 200 µL Wash Buffer

Add 25 µL Detection Antibodies per well

Incubate for 1 hour at RT

Do Not Aspirate

Add 25 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

Remove well contents and wash 2X with 200 µL Wash Buffer

Add 150 µL Sheath Fluid or Drive Fluid per well

Read on Luminex (100 µL, 50 beads per bead set)

PLATE WASHING

1.) Solid Plate
If using a solid plate, use either a hand-held magnet or magnetic plate washer.

A.) For hand-held magnet, rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 uL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

B.) For magnetic plate washer, let plate "soak" on magnet for 60 seconds to allow complete settling of the magnetic beads. Remove well contents by aspiration. Wash plate 2 times with 200 µL/well of Wash Buffer, letting beads "soak" for 60 seconds and removing Wash Buffer by aspiration after each wash. Repeat wash steps as recommended in Assay Procedure. **Note:** If using the recommended plate washer for magnetic beads (Bio-Tek ELx405) follow the appropriate equipment settings outlined on Page 14, see **EQUIPMENT SETTINGS.**

2.) Filter Plate (EMD Millipore Cat# MX-PLATE)
If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration after each wash Repeat wash steps as recommended in the Assay Procedure.

EQUIPMENT SETTINGS

Bio-Tek ELx405:

The general recommended wash protocol (Link Protocol) is as follows:

Soak Program:	Wash Program:
Soak →	Aspirate→Dispense→Soak→Aspirate→Dispense→Soak→Aspirate →Dispense→Soak→Aspirate

1.) Soak program:

1. Soak duration: 60 sec
2. Shake before soak?: NO

2.) Wash program:

Method:

1. Number of cycles: 2
2. soak/shake: YES
3. Soak duration: 60 sec
4. Shake before soak: NO
5. Prime after soak: NO

Dispense:

1. Dispense volume: 200 µL/well
2. Dispense flow rate: 5
3. Dispense height: 130 (16.51 mm)
4. Horizontal disp pos: 00 (0 mm)
5. Bottom Wash first?: NO
6. Prime before start?: NO

Aspiration:

1. Aspirate height: 35 (4.445 mm)
2. Horizontal Asp Pos: 30 (1.372 mm)
3. Aspiration rate: 06 (15.0 mm/sec)
4. Aspiration delay: 0
5. Crosswise Aspir: NO
6. Final Aspir: YES
7. Final Aspir delay: 0 (0 msec)

3.) Link program: (**Note:** this is the program to use during actual plate washing). Link together the Soak and Wash programs outlined above.

Note: After the final aspiration, there will be approximately 25 µl of residual Wash Buffer in each well. This is expected when using the BioTek Plate washer and this volume does not need to be aspirated from the plate.

If using an automatic plate washer other than BioTek ELx405, please refer to the manufacturers's recommendations for programming instructions.

These specifications are for the Luminex 200™, Luminex HTS and Luminex FLEXMAP 3D™ and MAGPIX® with xPonent software. Luminex instruments with other software (e.g. MasterPlex, StarStation, LiquiChip, Bio-Plex, LABScan100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex Magnetic Beads.

For magnetic bead assays, the Luminex 200™ and HTS instruments must be calibrated with the xPonent 3.1 compatible Calibration Kit (EMD Millipore Cat.# 40-275) and performance verified with the Performance Verification Kit (EMD Millipore Cat. # 40-276). The Luminex FLEXMap 3D™ instrument must be calibrated with the FLEXMAP 3D™ Calibrator Kit (EMD Millipore cat#40-028) and performance verified with the FLEXMAP 3D™ Performance Verification Kit (EMD Millipore cat#40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore cat# 40-049 and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore cat# 40-050).

NOTE: These assays cannot be run on Luminex 100™ instruments or any instruments using Luminex IS 2.3 or Luminex 1.7 software.

The Luminex probe height must be adjusted to the plate provided in the kit. Please use Cat.# MAG-PLATE, if additional plates are required for this purpose.

Events:	50, per bead		50, per bead	
Sample Size:	100 µL		100 µL	
Gate Settings	8,000 to 15,000			
Time Out	60 seconds			
Bead Set:	23-Plex Premix Beads		Customizable 23-Plex Beads	
	GM-CSF	14	GM-CSF	14
	TGFα	15	TGFα	15
	G-CSF	18	G-CSF	18
	IFNγ	20	IFNγ	20
	IL-2	33	IL-2	33
	IL-10	35	IL-10	35
	IL-15	37	IL-15	37
	sCD40L	38	sCD40L	38
	IL-17A	39	IL-17A	39
	IL-1ra	42	IL-1ra	42
	IL-13	44	IL-13	44
	IL-1β	46	IL-1β	46
	IL-4	53	IL-4	53
	IL-5	55	IL-5	55
	IL-6	57	IL-6	57
	IL-8	63	IL-8	63
	MIP-1α	65	MIP-1α	65
	MCP-1	67	MCP-1	67
	TNFα	72	TNFα	72
	MIP-1β	73	MIP-1β	73
	IL-12/23(p40)	74	IL-12/23(p40)	74
	VEGF	76	VEGF	76
	IL-18	78	IL-18	78

QUALITY CONTROLS

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located at the EMD Millipore website www.EMD Millipore.com/techlibrary/index.do using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Cross-Reactivity and Cross-Species Reactivity

There was no or negligible cross-reactivity among different analytes within the panel. However, the multiplexed assay panel has been tested with samples from multiple non-human primate species.

Detection of LPS or PHA Responses In PBMC From Multiple Non-Human Primate Species[1,2]

Cytokine	Chimpanzee	Baboon	Rhesus	Cynomolgus	Pig-Tail	African Green	Marmoset
IL-1ß	+++	+++	+++	+++	+++	+/-	+++
IL-2	+++	+++	+++	+++	+++	+++	NA
IL-4	+++	+++	+++	+++	+++	+++	-
IL-5	+++	+++	+++	+++	+++	+++	-
IL-6	+++	+++	+++	+++	+++	+++	-
TGFα	NA	-	+++	+++	NA	+++	NA
IL-8	+++	+++	+++	+++	+++	+++	-
IL-10[3]	+++	NA	+++	+++	NA	NA	NA
IL-12/23(p40)	+++	++	+++	++	++	++	-
IL-13	+++	+++	+++	+++	+++	+++	-
IL-15	+++	+++	+++	+++	+++	+++	-
IL-17A	+++	+++	+++	+++	+++	+++	+++
IL-18	+++	+++	+++	+++	+++	+++	-
IFNγ	+++	+++	+++	+++	+++	+++	-
G-CSF	+++	+++	+++	+++	+++	++	-
GM-CSF	+++	+++	+++	+++	+++	+++	-
TNFα	+++	+++	+++	+++	+++	+++	-
MCP-1	+++	+++	+++	+++	+++	+++	-
IL-1ra	+++	+++	+++	+++	+++	+++	+/-
sCD40L	+++	+++	+++	+++	+++	+++	-
MIP-1α	+++	+++	+++	+++	+++	+++	-
MIP-1ß	+++	+++	+++	+++	+++	++	+++
VEGF	+++	+++	+++	+++	+++	+++	+++

1. Tested samples are from PHA/LPS stimulated PBMC from two individual animals of each species, except IL-10
2. The "+++", "++", "+/-" or "-" indicate degree of reactivity with "+++" denotes strongly reacting and "-" denotes no detectable response in LPS- or PHA-stimulated PBMC cultures. “NA” indicates data not available.
3. IL-10 Chimpanzee, Rhesus and Cynomolgus were tested as LPS challenged serum.

Assay Sensitivities (minimum detectable concentrations, pg/mL)

MinDC: Minimum Detectable Concentration is calculated by the Milliplex Analyst Immunoassay Analysis Software from EMD Millipore, It is a measure of the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions.

Analyte	***MinDC[1] (pg/mL) Average + 2SD***
G-CSF	2.1
GM-CSF	1.8
IFNγ	1.6
IL-1β	1.2
IL-1ra	2.4
IL-2	2.1
IL-4	3.1
IL-5	1.5
IL-6	1.6
IL-8	1.1
IL-10	6.4
IL-12/23(p40)	1.5
IL-13	5.8
IL-15	0.5
IL-17A	1.3
MCP-1	3.1
MIP-1β	1.6
MIP-1α	4.9
sCD40L	2.1
TGFα	1.1
TNFα	1.6
VEGF	13.6
IL-18	6.1

Precision

Intra-assay precision is generated from the mean of the %CV's from 8 reportable results across two different concentrations of analytes in a single assay. Inter-assay precision is generated from the mean of the %CV's across two different concentrations of analytes across 6 different assays.

Analyte	***Intra-Assay (CV%)***	***Inter-Assay (CV%)***
G-CSF	< 5	< 15
GM-CSF	< 5	< 15
IFNγ	< 5	< 15
IL-1β	< 5	< 15
IL-1ra	< 5	< 15
IL-2	< 5	< 15
IL-4	< 5	< 15
IL-5	< 5	< 15
IL-6	< 5	< 15
IL-8	< 5	< 15
IL-10	< 5	< 15
IL-12/23(p40)	< 5	< 15
IL-13	< 5	< 15
IL-15	< 5	< 15
IL-17A	< 5	< 15
MCP-1	< 5	< 15
MIP-1β	< 5	< 15
MIP-1α	< 5	< 15
sCD40L	< 5	< 15
TGFα	< 5	< 15
TNFα	< 5	< 15
VEGF	< 5	< 15
IL-18	< 5	< 15

Accuracy

Spike Recovery: The data represent mean percent recovery of spiked standards ranging from low, medium, and high concentration in serum matrix samples (n=6).

Analyte	Recovery
G-CSF	93
GM-CSF	99
IFNγ	92
IL-1β	91
IL-1ra	90
IL-2	90
IL-4	94
IL-5	96
IL-6	87
IL-8	89
IL-10	101
IL-12/23(p40)	95
IL-13	90
IL-15	93
IL-17A	93
MCP-1	90
MIP-1β	90
MIP-1α	89
sCD40L	90
TGFα	98
TNFα	96
VEGF	70
IL-18	91

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Insufficient Bead Count	Plate Washer aspirate height set too low	Adjust aspiration height according to manufacturers instructions.
	Bead mix prepared inappropriately	Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting this into the plate.
	Samples cause interference due to particulate matter or viscosity	See above. Also sample probe may need to be cleaned with Alcohol flush, Back flush and washes; or if needed probe should be removed and sonicated.
	Probe height not adjusted correctly	When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 4 alignment discs. When reading the assay on FLEXMAP 3D™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc. When reading the assay on MAGPIX, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 2 alignment disc.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately, and pipeting with Multichannel pipets without touching reagent in plate.
	Matrix used has endogenous analyte or interference	Check matrix ingredients for cross reacting components (e.g. interleukin modified tissue culture medium).
	Insufficient washes	Increase number of washes.
Beads not in region or gate	Luminex not calibrated correctly or recently	Calibrate Luminex based on Instrument Manufacturer's instructions, at least once a week or if temperature has changed by >3^{o}C.
	Gate Settings not adjusted correctly	Some Luminex instruments (e.g. Bioplex) require different gate settings than those described in the Kit protocol. Use Instrument default settings.
	Wrong bead regions in protocol template	Check kit protocol for correct bead regions or analyte selection.
	Incorrect sample type used	Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required.
	Instrument not washed or primed	Prime the Luminex 4 times to rid of air bubbles, wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid.

	Beads were exposed to light	Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.
Signal for whole plate is same as background	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue.
	Streptavidin-Phycoerythrin was not added	Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been removed, sensitivity may be low.
Low signal for standard curve	Detection Antibody may have been removed prior to adding Streptavidin Phycoerythrin	May need to repeat assay if desired sensitivity not achieved.
	Incubations done at inappropriate temperatures, timings or agitation	Assay conditions need to be checked.
Signals too high, standard curves are saturated	Calibration target value set too high	With some Luminex Instrument (e.g. Bio-plex) Default target setting for RP1 calibrator is set at High PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with standard curve and samples	Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte	If below detectable levels, it may be possible to use higher sample volume. Check with tech support for appropriate protocol modifications.
	Samples contain analyte concentrations higher than highest standard point.	Samples may require dilution and reanalysis for just that particular analyte.
	Standard curve was saturated at higher end of curve.	See above.
High Variation in samples and/or standards	Multichannel pipet may not be calibrated	Calibrate pipets.
	Plate washing was not uniform	Confirm all reagents are removed completely in all wash steps.
	Samples may have high particulate matter or other interfering substances	See above.
	Plate agitation was insufficient	Plate should be agitated during all incubation steps using a vertical plate shaker at a speed where beads are in constant motion without causing splashing.
	Cross well contamination	Check when reusing plate sealer that no reagent has touched sealer. Care should be taken when using same pipet tips that are used for reagent additions and that pipet tip does not touch reagent in plate.
FOR FILTER PLATES ONLY		
Filter plate will not vacuum	Vacuum pressure is insufficient	Increase vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble	Centrifuge samples just prior to assay setup

	particles	and use supernatant.
	High lipid concentration	After centrifugation, remove lipid layer and use supernatant.
Plate leaked	Vacuum Pressure too high	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (blocked) plate and continue.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate holder or raised edge so bottom of filter is not touching any surface.
	Insufficient blotting of filter plate bottom causing wicking	Blot the bottom of the filter plate well with absorbent towels after each wash step.
	Pipette touching plate filter during additions	Pipette to the side of plate.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
	Sample too viscous	May need to dilute sample.

REPLACEMENT REAGENTS	**Cat #**
Non-Human Primate Cytokine Standard	MXPR8040
Non-Human Primate Cytokine Quality Controls	MXPR6040
Serum Matrix	MXPRSM
Non-human Primate Cytokine Detection Antibodies	MXPR1040-2
Streptavidin-Phycoerythrin	L-SAPE4
Assay Buffer	L-AB
Bead Diluent	LBD
Set of two 96-Well Plate with 2 sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Beads

Cytokine	*Bead #*	*Cat. #*
G-CSF	18	HGCSF-MAG
GM-CSF	14	PRGMCSF-MAG
IFNγ	20	PRIFNG-MAG
IL-1β	46	HCYIL1B-MAG
IL-1ra	42	HIL1RA-MAG
IL-2	33	PRIL2-MAG
IL-4	53	HIL4-MAG
IL-5	55	HIL5-MAG
IL-6	57	HCYIL6-MAG
IL-8	63	HCYIL8-MAG
IL-10	35	PRIL10-MAG
IL-12/23(p40)	74	PRIL12P40-MAG
IL-13	44	PRIL13-MAG
IL-15	37	HIL15-MAG
IL-17A	39	HIL17-MAG
IL-18	78	PRIL18-MAG
MCP-1	67	HCYMCP1-MAG
MIP-1β	73	HMIP1B-MAG
MIP-1α	65	PRMIP1A-MAG
sCD40L	38	HCD40L-MAG
TGFα	15	HCYTGFA-MAG
TNFα	72	PRTNFA-MAG
VEGF	76	PRVEGF-MAG
Premixed 23-plex Beads		PRCYPMX23-MAG

ORDERING INFORMATION

To place an order:

To assure the clarity of your custom kit order, please FAX the following information to our customer service department:

- Your name, telephone and/or fax number
- Customer account number
- Shipping and billing address
- Purchase order number
- Catalog number and description of product
- Quantity of kits
- Selection of MILLIPLEX MAP Cytokine Analytes/Serum Matrix Requirements

FAX: (636) 441-8050
Toll Free US: (800) EMD MILLIPORE

MAIL ORDERS: EMD Millipore Corp.
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

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Material Safety Data Sheets (MSDS)

Material Safety Data Sheets for EMD Millipore products may be ordered by fax or phone or through our website at www.EMD Millipore.com/techlibrary/index.do

WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/mL Standard (Background)	Standard 4	QC-1 Control	Etc.								
B	0 pg/mL Standard (Background)	Standard 4	QC-1 Control									
C	Standard 1	Standard 5	QC-2 Control									
D	Standard 1	Standard 5	QC-2 Control									
E	Standard 2	Standard 6	Sample 1									
F	Standard 2	Standard 6	Sample 1									
G	Standard 3	Standard 7	Sample 2									
H	Standard 3	Standard 7	Sample 2									