Non-Human Primate Cytokine

96-Well Plate Assay

Cat. # MPXPRCYTO-40K

MILLIPLEX®MAP

NON-HUMAN PRIMATE CYTOKINE KIT
96 Well Plate Assay

#MPXPRCYTO-40K

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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® 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.

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

- MILLIPLEX® MAP offers you the ability to:
  - 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® Non-Human Primate Cytokine kit is to be used for the simultaneous quantification of the following 23 non-human primate cytokines and chemokines: 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-17, IL-18, IFNγ, G-CSF, GM-CSF, MCP-1, MIP-1α, MIP-1β, TNFα, TGFα, Soluble CD40 Ligand (sCD40L), and VEGF. This kit may be used for the analysis of all above cytokines and chemokines in tissue / cell lysate and culture supernatant samples. This kit may also be used in serum, plasma, other body fluids, and tissue extract samples for the analysis of all above cytokines and chemokines.

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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-17	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 is based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life sciences, and is capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded beads known as 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 covalently coupled 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-phycoerythrin (SAPE) conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- The microspheres are allowed to pass rapidly through a laser, that excites the internal dyes identifying 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.
- Once the standards and controls have been reconstituted, immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSITUTED STANDARDS OR CONTROLS IN GLASS VIALS**. 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 Filter Plate with 2 sealers	MX-PLATE		1 plate 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	1 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 pre-mixed panel)	----------	----------	1 bottle

Included Non-Human Primate Cytokine Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel (see following table page 5).

Non-Human Primate Cytokine Antibody-Immobilized Beads:

Bead/Analyte Name	Luminex Bead Region	Customizable 23 Analytes (50X Concentration, 90 µL)	
		Available	Cat. #
Anti- G-CSF Bead	12	✓	MXHGCSF
Anti- GM-CSF Bead	15	✓	MXPRGM-CSF
Anti- IFNγ Bead	17	✓	MXPRIFN-G
Anti- IL-1β Bead	24	✓	MXHIL-1B
Anti- IL-1ra Bead	26	✓	MXHIL-1RA
Anti- IL-2 Bead	28	✓	MXPRIL-2
Anti- IL-4 Bead	32	✓	MXHIL-4
Anti- IL-5 Bead	34	✓	MXHIL-5
Anti- IL-6 Bead	36	✓	MXHIL-6
Anti- IL-8 Bead	40	✓	MXHIL-8
Anti- IL-10 Bead	44	✓	PRIL-10
Anti- IL-12/23 (p40) Bead	47	✓	MXPRIL-12
Anti- IL-13 Bead	49	✓	MXPRIL-13
Anti- IL-15 Bead	52	✓	MXHIL-15
Anti- IL-17 Bead	54	✓	MXHIL-17
Anti- MCP-1 Bead	58	✓	MXHMCP-1
Anti- MIP-1β Bead	66	✓	MXHMIP-1B
Anti- MIP-1α Bead	71	✓	MXPRMIP-1A
Anti- sCD40L Bead	74	✓	MXHCD40L
Anti-TGFα Bead	78	✓	MXHTGF-A
Anti- TNFα Bead	81	✓	MXPRTNF-A
Anti- VEGF Bead	84	✓	MXPRVEGF
Anti- IL-18 Bead	86	✓	MXPRIL-18

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (Luminex Catalogue # 40-50000)

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. Aluminum Foil
6. Rubber Bands
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. Vacuum Filtration Unit (EMD Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with EMD Millipore Vacuum Pump Catalog #WP6111560 or equivalent)
12. Luminex$^{100, 200, HTS}$ by Luminex Corporation
13. Plate Holder (EMD Millipore Catalog # MX-STAND)

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 beads with opaque plate lid or aluminum foil during all incubation steps. **If the opaque lid is used as the plate holder, wrap the plate holder and plate with aluminum foil.**
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- The bottom of the Microtiter Filter Plate should not be in direct contact with any surface during assay set-up or incubation times. The plate can be set on a plate holder or on the non-flat side of the plate cover or any other plate holder to raise the plate from the surface. A plate holder can be purchased separately from EMD Millipore (EMD Millipore Catalog #MX-STAND).
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- After the wash steps, keep the bottom of the Microtiter Filter Plate clean by blotting on paper towels or absorbent pads to prevent any leakage due to capillary action.
- Keep the vacuum suction on the plate as low as possible. It is recommended to have a vacuum setting that will remove 200 µL of buffer in $\geq$ 5 seconds (equivalent to < 100 mmHg).
- After hydration, all standards and controls must be transferred to polypropylene tubes.
- 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 Bead Mix 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 cytokines and chemokines.

- 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. Adjust probe height according to the protocols recommended by Luminex to the kit filter plate using 3 alignment discs prior to reading an assay.
- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in blank, standard curve and control wells. If samples are diluted in Assay Buffer, use the Assay Buffer as the matrix solution.
- For serum / plasma samples requiring dilutions, use the Serum Matrix provided in the kit as the sample diluent.
- 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 blood to clot for at least 30 minutes, centrifuge the clotted blood for 10 minutes at 1000 xg. Remove serum and assay immediately or aliquot and store samples at ≤ -20ºC.
- Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge to remove particulates prior to use in the assay.
- Use Serum Matrix as the diluent for quantifying cytokines/chemokines in serum or plasma 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 1000 xg within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20ºC.
- Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge to remove particulates prior to use in the assay.
- Use Serum Matrix as the diluent for quantifying cytokines/chemokines in serum or plasma 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 ≤ -20ºC.
- Avoid multiple (>2) freeze/thaw cycles.
- Tissue culture supernatant may require a dilution with an appropriate control medium prior to assay.

NOTE:

- A maximum of 25 µL per well of neat or diluted 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

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.

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 and then transfer the controls to appropriately labeled polypropylene microfuge tubes. Unused portions 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 30 mL of 10X Wash Buffer with 270 mL deionized water. Store unused portions 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.

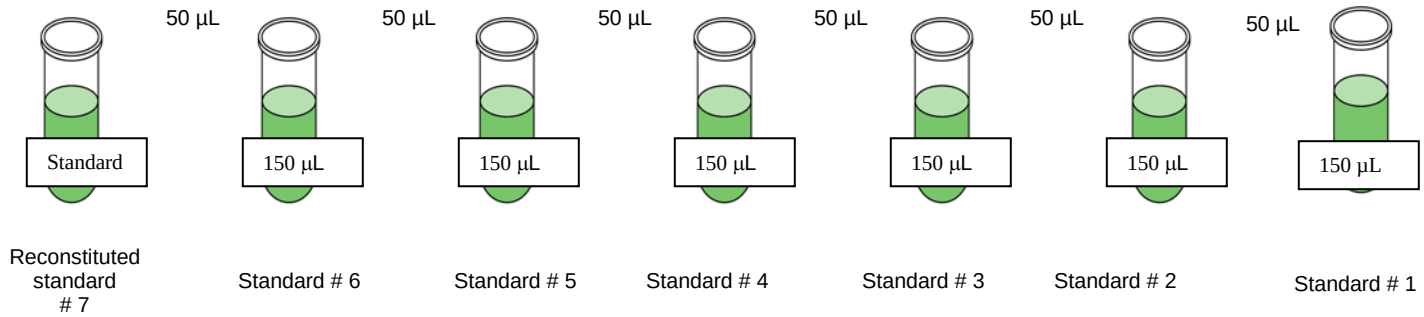
PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

E. Preparation of Non-Human Primate Cytokine 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, vortex and transfer the contents to a polypropylene microfuge tube labeled “Standard

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 Control) 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



PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

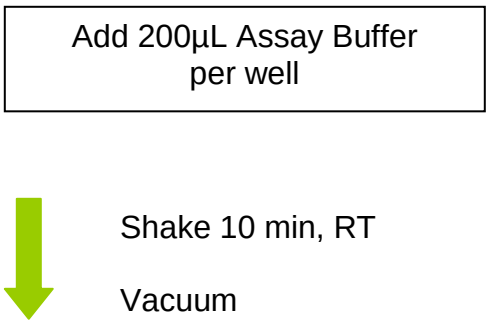
After serial dilutions, the tubes should have the following concentrations for constructing standard curves.

Standard Tube #	IL-4 (pg/ml)	IL-10, IL-18 (pg/ml)	All Other Analytes
1	4.9	12.2	2.4
2	19.5	48.8	9.8
3	78.1	195.3	39.0
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 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, 1, 2, 3, 4, 5, 6, and 7 pg/mL), Controls 1 and 2, and samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.
- 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. Block the filter plate by pipetting 200 µL of Assay Buffer into each well of the microtiter plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Remove Assay Buffer by vacuum. **(NOTE: DO NOT INVERT PLATE.)** Blot excess Assay Buffer from the bottom of the plate with an absorbent pad or paper towels.
3. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 standard (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 samples, use proper control culture medium as the matrix solution.
6. Add 25 µL of sample into the appropriate wells.
7. Vortex Bead Bottle and add 25 µL of the mixed or Premixed Beads to each well. (Note: During addition of mixed Beads, shake bead mix intermittently to avoid settling)
8. Seal, the plate with a plate sealer, cover it with the opaque lid. Wrap a rubber band around the plate holder, plate and lid combination and incubate with agitation on a plate shaker for overnight incubation at 4°C or 2 hour at room temperature (20-25°C). *An overnight incubation (16-18 hr) may improve assay sensitivity for some analytes.* **Same protocol should be followed for multiple assays in a study.**
9. Gently remove fluid by vacuum. **(NOTE: DO NOT INVERT PLATE)**.
10. Wash plate 2 times with 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration between each wash. Blot excess Wash Buffer from the bottom the plate by with an absorbent pad or paper towels.
11. Add 25 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)
12. Seal, cover with lid, and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT VACUUM AFTER INCUBATION**
13. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.

- Add 25µL Standard, Control, to appropriate wells
- Add 25 µL Assay Buffer to the Background and sample wells
- Add 25µL Samples to sample wells
- Add 25µL Appropriate Matrix to background, standards, control wells
- Add 25µL Beads to each well

Incubate overnight at 4°C or 2 hour at RT with shaking.

Vacuum. Wash 2X with 200µL Wash Buffer

Add 25µL Detection Antibody per well

Incubate 1 hour at RT with shaking.

Do Not Vacuum

Add 25µL Streptavidin-Phycoerythrin per well

14. Seal, cover with lid and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
15. Gently remove all contents by vacuum. **(NOTE: DO NOT INVERT PLATE)**.
16. Wash plate 2 times with 200 µL Wash Buffer per well, removing Wash Buffer by vacuum filtration between each wash. Wipe any excess buffer on the bottom of the plate with a tissue.
17. Add 150 µL of Sheath Fluid to all wells. Resuspend the beads on a plate shaker for 5 minutes.
18. Run plate on Luminex$^{100, 200, HTS}$.
19. Save and analyze the median Fluorescent Intensity (MFI) data using a weighted 5-parameter logistic or a smoothed spline curve-fitting method for calculating analyte concentrations in samples. If samples are diluted, multiply the result by the dilution factor.

Incubate 30 min at RT with shaking.

Vacuum and Wash 2X with 200 µL Wash Buffer

Add 150µL Sheath Fluid per well

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

EQUIPMENT SETTINGS

These specifications are for the Luminex^{100} v.1.7 or Luminex^{100} IS v2.1, IS 2.2, Luminex^{200} v2.3, xPONENT, and Luminex ^{HTS}. 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.

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:	Customizable 23-Plex Beads	
	G-CSF	12
	GM-CSF	15
	IFNγ	17
	IL-1β	24
	IL-1ra	26
	IL-2	28
	IL-4	32
	IL-5	34
	IL-6	36
	IL-8	40
	IL-10	44
	IL-12/23(p40)	47
	IL-13	49
	IL-15	52
	IL-17	54
	MCP-1	58
	MIP-1β	66
	MIP-1α	71
	sCD40L	74
	TGFα	78
	TNFα	81
	VEGF	84
	IL-18	86

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.millipore.com/techlibrary/index.do using the catalog numbers as the keyword.

ASSAY CHARACTERISTICS*

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	2.2
GM-CSF	1.4
IFNγ	2.2
IL-1β	1.4
IL-1ra	2.3
IL-2	6.6
IL-4	2.7
IL-5	2.3
IL-6	1.6
IL-8	2.5
IL-10	6.2
IL-12/23 (p40)	1.2
IL-13	3.5
IL-15	1.7
IL-17	1.7
MCP-1	1.6
MIP-1β	5.1
MIP-1α	3.7
sCD40L	1.9
TGFα	1.7
TNFα	2.1
VEGF	13.5
IL-18	7.3

1. MinDc: Minimum Detectable Concentration

***Assay performance characteristics generally vary with specific assay conditions, which change from laboratory to laboratory. The data in this section is for reference only.**

ASSAY CHARACTERISTICS (continued)

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-17	+++	+++	+++	+++	+++	+++	+++
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 in LPS challenged serum.

Precision

Intra-assay precision is generated from the mean of the %CV's from 8 reportable results across two different concentration of cytokines in one experiment. Inter-assay precision is generated from the mean of the %CV's from two reportable results each for two different concentrations of cytokine across 4 different experiments.

Analyte	***Intra-Assay (CV%)***	***Inter-Assay (CV%)***
G-CSF	5.6	10.1
GM-CSF	5.1	17.5
IFNγ	5.5	10.2
IL-1β	5.6	10.4
IL-1ra	4.7	9.2
IL-2	4.9	11.8
IL-4	4.5	7.8
IL-5	4.6	8.9
IL-6	5.0	9.7
IL-8	5.0	8.0
IL-10	5.0	6.0
IL-12/23 (p40)	5.3	6.9
IL-13	5.4	8.8
IL-15	4.4	8.5
IL-17	5.8	9.9
MCP-1	5.3	12.9
MIP-1β	8.2	12.6
MIP-1α	5.4	10.1
sCD40L	6.9	12.5
TGFα	6.0	10.8
TNFα	4.6	11.4
VEGF	7.8	12.4
IL-18	6.3	14.4

ASSAY CHARACTERISTICS (continued)

Accuracy

Spike Recovery: The data represents the average of 3 levels of analytes recovered in serum matrix.

Analyte	Recovery%
G-CSF	100.6
GM-CSF	98.8
IFNγ	99.6
IL-1β	101.2
IL-1ra	97.6
IL-2	97.4
IL-4	99.3
IL-5	99.8
IL-6	98.4
IL-8	98.9
IL-10	100.0
IL-12/23 (p40)	96.5
IL-13	97.9
IL-15	99.3
IL-17	99.4
MCP-1	102.5
MIP-1β	91.1
MIP-1α	95.0
sCD40L	97.9
TGFα	102.0
TNFα	99.9
VEGF	97.6
IL-18	108.3

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Filter plate will not vacuum	Vacuum pressure is insufficient	Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble particles	Centrifuge samples just prior to assay set-up and use supernatant. If sample lipid concentration is high, centrifuge and remove lipid layer and then use the supernatant.
	Sample too viscous	May need to dilute sample
Insufficient bead count	Vacuum pressure too high	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds.
	Bead mix prepared improperly	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	Adjust probe to 3 alignment discs in well H6.
Plate leaked	Vacuum Pressure too high	Adjust vacuum pressure such that 0.2 mL 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 caused 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.
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 vacuumed out, sensitivity may be low.
Low signal for standard curve	Detection Antibody may have been vacuumed out 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	Multichannel pipet may not be calibrated	Calibrate pipets.

standards	Plate washing was not uniform	Confirm all reagents are vacuumed out 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.

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 Filter Plates with Sealers	MX-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Beads

Cytokine	*Bead #*	*Cat. #*
G-CSF	*12*	*MXHGCSF*
GM-CSF	*15*	*MXPRGM-CSF*
IFNγ	*17*	*MXPRIFN-G*
IL-1β	*24*	*MXHIL-1B*
IL-1ra	*26*	*MXHIL-1RA*
IL-2	*28*	*MXPRIL-2*
IL-4	*32*	*MXHIL-4*
IL-5	*34*	*MXHIL-5*
IL-6	*36*	*MXHIL-6*
IL-8	*40*	*MXHIL-8*
IL-10	*44*	*PRIL-10*
IL-12/23(p40)	*47*	*MXPRIL-12*
IL-13	*49*	*MXPRIL-13*
IL-15	*52*	*MXHIL-15*
IL-17	*54*	*MXHIL-17*
MCP-1	*58*	*MXHMCP-1*
MIP-1β	*66*	*MXHMIP-1B*
MIP-1α	*71*	*MXPRMIP-1A*
sCD40L	*74*	*MXHCD40L*
TGFα	*78*	*MXHTGF-A*
TNFα	*81*	*MXPRTNF-A*
VEGF	*84*	*MXPRVEGF*
IL-18	*86*	*MXPRIL-18*

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WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 ng/mL Standard (Background)	Standard 4	QC-1 Control	Etc.								
B	0 ng/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									