



**Human Cytokine /
Chemokine**

96-Well Plate Assay

**Cat. # MPXHCYTO-60K,
MPXHCYTO60KPMX14,
MPXHCYTO60KPMX26,
MPXHCYTO60KPMX39, or
MPXHCYTO60KPMX42**

MILLIPLEX[®] MAP

HUMAN CYTOKINE / CHEMOKINE KIT 96 Well Plate Assay

**#MPXHCYTO-60K or
#MPXHCYTO60KPMX14 (premixed) or
#MPXHCYTO60KPMX26 (premixed) or
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#MPXHCYTO60KPMX42 (premixed)**

<u>TABLE OF CONTENTS</u>	<u>PAGE</u>
Introduction	2
Principle	3
Storage Conditions Upon Receipt	3
Reagents Supplied	4
Materials Required But Not Provided	6
Safety Precautions	6
Technical Guidelines	6
Sample Collection And Storage	8
Preparation of Reagents for Immunoassay	9
Immunoassay Procedure	11
Equipment Settings	13
Quality Controls	14
Assay Characteristics	14
Troubleshooting Guide	18
Replacement Reagents	21
Ordering Information	22
Well Map	23

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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 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™ Human Cytokine / Chemokine 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.

Millipore's MILLIPLEX® Human Cytokine / Chemokine panel is the most versatile system available for cytokine and chemokine research.

- MILLIPLEX® MAP offers you the ability to:
 - Select a premixed kit (14-, 26-, 39- or 42-plex).
 - Choose any combination of analytes from our panel of 42 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.

Millipore's MILLIPLEX® Human Cytokine / Chemokine kit is to be used for the simultaneous quantification of the following 42 human cytokines and chemokines: EGF, Eotaxin, FGF-2, Flt-3 Ligand, Fractalkine, G-CSF, GM-CSF, GRO, IFN α 2, IFN γ , IL-1ra, IL-1 α , IL-1 β , IL-2, sIL-2R α , IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17A, IP-10, MCP-1, MCP-3, MDC, MIP-1 α , MIP-1 β , PDGF-AA, PDGF-AB/BB, RANTES, sCD40L, TGF α , TNF α , TNF β , and VEGF.

This kit may be used for the analysis of all or any combination of the above cytokines and chemokines in tissue/cell lysate and culture supernatant samples. This kit can also be used in serum or plasma samples for the analysis of all or any combination of the above cytokines and chemokines except RANTES, PDGF-AA, and PDGF-AB/BB because of high concentrations of the three analytes in the blood. **Serum or plasma samples should be diluted 1:100 when the kit is used for analyzing RANTES, PDGF-AA, and PDGF-AB/BB.**

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.

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 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 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.
- Once the standards and controls have been reconstituted, immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSTITUTED 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 Antibodies, and Streptavidin-Phycoerythrin.**

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8 °C

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
Human Cytokine / Chemokine Standard	MXH8060 or MXH8060-2	lyophilized	1 vial
Human Cytokine Quality Controls 1 and 2	MXH6060 or MXH6060-2	lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	MXHSM	lyophilized	1 vial (required for serum and plasma samples only)
Set of one 96-Well Filter Plate with 2 Sealers	MX-PLATE		1 plate 2 sealers
Assay Buffer	L-AB	30 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	1 bottle
Human Cytokine Detection Antibodies	MXH1060-1 or MXH1060-2 or MXH1060-3 or MXH1060-4	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE9 <i>(Use with Cat. # MXH1060-1)</i> or L-SAPE3 <i>(Use with Cat. # MXH1060-2)</i> or L-SAPE10 <i>(Use with Cat. # MXH1060-3)</i> or L-SAPE11 <i>(Use with Cat. # MXH1060-4)</i>	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

Human Cytokine / Chemokine Antibody-Immobilized Premixed Beads:

Premixed 14-plex Beads	MXHPMX14	3.5 mL	1 bottle
Premixed 26-plex Beads	MXHPMX26	3.5 mL	1 bottle
Premixed 39-plex Beads	MXHPMX39	3.5 mL	1 bottle
Premixed 42-plex Beads (tissue culture only)	MXHPMX39, MXHRNTS, MXHPDGFAA, MXHPDGFAB-BB	3.5 mL 90 µL 90 µL 90 µL	1 bottle + 3 bead vials

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

Human Cytokine / Chemokine Antibody-Immobilized Beads:

Bead/Analyte Name	Luminex Bead Region	Customizable 42 Analytes (50X concentration, 90µL) Available		14-Plex Premixed Beads	26-Plex Premixed Beads	39-Plex Premixed Beads
			Cat. #			
Anti-Human EGF Bead	2	<input type="checkbox"/>	MXHEGF			<input type="checkbox"/>
Anti-Human Eotaxin Bead	4	<input type="checkbox"/>	MXHETXN		<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human FGF-2 Bead	6	<input type="checkbox"/>	MXHFGF2			<input type="checkbox"/>
Anti-Human Flt-3 Ligand Bead	8	<input type="checkbox"/>	MXHFLT3L			<input type="checkbox"/>
Anti-Human Fractalkine Bead	10	<input type="checkbox"/>	MXHFKN			<input type="checkbox"/>
Anti-Human G-CSF Bead	12	<input type="checkbox"/>	MXHGCSF		<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human GM-CSF Bead	14	<input type="checkbox"/>	MXHGMCSF	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human GRO Bead	16	<input type="checkbox"/>	MXHGRO			<input type="checkbox"/>
Anti-Human IFNα2 Bead	18	<input type="checkbox"/>	MXHIFNA2		<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IFNγ Bead	20	<input type="checkbox"/>	MXHIFNG	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-1α Bead	22	<input type="checkbox"/>	MXHIL-1A		<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-1β Bead	24	<input type="checkbox"/>	MXHIL-1B	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-1ra Bead	26	<input type="checkbox"/>	MXHIL-1RA			<input type="checkbox"/>
Anti-Human IL-2 Bead	28	<input type="checkbox"/>	MXHIL-2	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-3 Bead	30	<input type="checkbox"/>	MXHIL-3		<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-4 Bead	32	<input type="checkbox"/>	MXHIL-4	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-5 Bead	34	<input type="checkbox"/>	MXHIL-5	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-6 Bead	36	<input type="checkbox"/>	MXHIL-6	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-7 Bead	38	<input type="checkbox"/>	MXHIL-7	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-8 Bead	40	<input type="checkbox"/>	MXHIL-8	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-9 Bead	42	<input type="checkbox"/>	MXHIL-9			<input type="checkbox"/>
Anti-Human IL-10 Bead	44	<input type="checkbox"/>	MXHIL-10	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-12 (p40) Bead	46	<input type="checkbox"/>	MXH12P40		<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-12 (p70) Bead	48	<input type="checkbox"/>	MXH12P70	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-13 Bead	50	<input type="checkbox"/>	MXHIL-13	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-15 Bead	52	<input type="checkbox"/>	MXHIL-15		<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IL-17A Bead	54	<input type="checkbox"/>	MXHIL-17A		<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human IP-10 Bead	56	<input type="checkbox"/>	MXHIP10		<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human MCP-1 Bead	58	<input type="checkbox"/>	MXHMCP-1	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human MCP-3 Bead	60	<input type="checkbox"/>	MXHMCP3			<input type="checkbox"/>
Anti-Human MDC Bead	62	<input type="checkbox"/>	MXHMDC			<input type="checkbox"/>
Anti-Human MIP-1α Bead	64	<input type="checkbox"/>	MXHMIP-1A		<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human MIP-1β Bead	66	<input type="checkbox"/>	MXHMIP-1B		<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human PDGF-AA Bead	68	<input type="checkbox"/>	MXHPDGFAA			
Anti-Human PDGF-AB/BB Bead	70	<input type="checkbox"/>	MXHPDGFAB-BB			
Anti-Human RANTES Bead	72	<input type="checkbox"/>	MXHRNTS			
Anti-Human sCD40L Bead	74	<input type="checkbox"/>	MXHCD40L			<input type="checkbox"/>
Anti-Human sIL-2Rα Bead	76	<input type="checkbox"/>	MXHIL-2RA			<input type="checkbox"/>
Anti-Human TGFα Bead	78	<input type="checkbox"/>	MXHTGF-A			<input type="checkbox"/>
Anti-Human TNFα Bead	80	<input type="checkbox"/>	MXHTNF-A	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human TNFβ Bead	82	<input type="checkbox"/>	MXHTNF-B		<input type="checkbox"/>	<input type="checkbox"/>
Anti-Human VEGF Bead	86	<input type="checkbox"/>	MXHVEGF			<input type="checkbox"/>

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 μ L to 1000 μ L
2. Multichannel Pipettes capable of delivering 5 μ L to 50 μ L or 25 μ L to 200 μ L
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 (Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with Millipore Vacuum Pump Catalog #WP6111560 or equivalent)
12. Luminex 100™ IS, 200™, or HTS by Luminex Corporation
13. Plate Stand (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.
- 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 stand or on the non-flat side of the plate cover or any other plate holder to raise the plate from the surface. A plate stand can be purchased separately from Millipore (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 μ L 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 $\leq -20^{\circ}\text{C}$ for 1 month and at $\leq -80^{\circ}\text{C}$ 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 $2-8^{\circ}\text{C}$ 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 $2-8^{\circ}\text{C}$ 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 background, standard curve and control wells. If samples are diluted in Assay Buffer, use the Assay Buffer as matrix.
- For serum/plasma samples, use the Serum Matrix provided in the kit.
- 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 $\leq -20^{\circ}\text{C}$.
- 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.
- For all analytes **except** RANTES, PDGF-AA, or PDGF-AB/BB, use the Serum Matrix as the sample diluent if the serum sample requires dilution. Additional Serum Matrix can be purchased from Millipore (Millipore Catalog #MXHSM).
- **When RANTES, PDGF-AA, and/or PDGF-AB/BB are measured**, serum samples should be diluted 1:100 in the Assay Buffer and a standard curve with Assay Buffer matrix should be used accordingly. If serum samples require further dilution beyond 1:100, continue to use Assay Buffer 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 $\leq -20^{\circ}\text{C}$.
- 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.
- For all analytes **except** RANTES, PDGF-AA, or PDGF-AB/BB, use the Serum Matrix as the sample diluent if the plasma sample requires dilution. Additional Serum Matrix can be purchased from Millipore (Millipore Catalog #MXHSM).
- **When RANTES, PDGF-AA, and/or PDGF-AB/BB are measured**, plasma samples should be diluted 1:100 in the Assay Buffer and a standard curve with Assay Buffer matrix should be used accordingly. If plasma samples require further dilution beyond 1:100, continue to use Assay Buffer 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 $\leq -20^{\circ}\text{C}$.
- 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

If premixed beads are used, sonicate the premixed bead bottle 30 seconds and then vortex for 1 minute before use.

To prepare 40-, 41- or 42-plex premixed beads, add 70 μL of each additional bead (RANTES, PDGF-AA and/or PDGF-AB/BB) to the 39-plex premixed beads. Mix well before use.

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 portion may be stored at 2-8°C for up to one month.

Example 1: When using 30 cytokine antibody-immobilized beads, add 60 μL from each of the 30 bead sets to the Mixing Bottle. Then add 1.2 mL Bead Diluent.

Example 2: When using 9 cytokine antibody-immobilized beads, add 60 μL from each of the 9 bead sets to the Mixing Bottle. Then add 2.46 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 portion may be stored at $\leq -20^\circ\text{C}$ 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 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 $\leq -20^{\circ}\text{C}$ for up to one month.

E. Preparation of Human Cytokine Standard

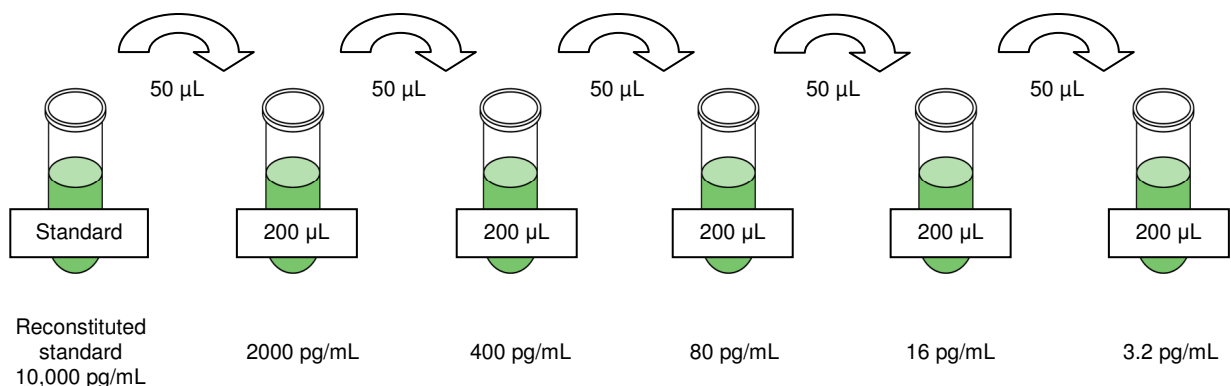
1.) Prior to use, reconstitute the Human Cytokine Standard with 250 μL deionized water to give a 10,000 pg/mL concentration of standard for all analytes. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes and then transfer the standard to an appropriately labeled polypropylene microfuge tube. This will be used as the 10,000 pg/mL standard; the unused portion may be stored at $\leq -20^{\circ}\text{C}$ for up to one month.

2.) Preparation of Working Standards

Label five polypropylene microfuge tubes 2000, 400, 80, 16, and 3.2 pg/mL. Add 200 μL of Assay Buffer to each of the five tubes. Prepare serial dilutions by adding 50 μL of the 10,000 pg/mL reconstituted standard to the 2000 pg/mL tube, mix well and transfer 50 μL of the 2000 pg/mL standard to the 400 pg/mL tube, mix well and transfer 50 μL of the 400 pg/mL standard to the 80 pg/mL tube, mix well and transfer 50 μL of the 80 pg/mL standard to 16 pg/mL tube, mix well and transfer 50 μL of the 16 pg/mL standard to the 3.2 pg/mL tube and mix well. The 0 pg/mL standard (Background) will be Assay Buffer

Standard Concentration (pg/mL)	Volume of Deionized Water to Add	Volume of Standard to Add
10,000	250 μL	0

Standard Concentration (pg/mL)	Volume of Assay Buffer to Add	Volume of Standard to Add
2000	200 μL	50 μL of 10,000 pg/mL
400	200 μL	50 μL of 2000 pg/mL
80	200 μL	50 μL of 400 pg/mL
16	200 μL	50 μL of 80 pg/mL
3.2	200 μL	50 μL of 16 pg/mL



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), 3.2, 16, 80, 400, 2000, and 10,000 pg/mL], 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.
- 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. Prewet the filter plate by pipetting 200 μ L of Assay Buffer into each well of the Microtiter Filter 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 μ L of each Standard or Control into the appropriate wells. Assay Buffer should be used for the 0 pg/mL standard (Background).
4. Add 25 μ L of Assay Buffer to the sample wells.
5. Add 25 μ L 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 μ L of Sample into the appropriate wells.
7. Vortex Mixing Bottle and add 25 μ L of the Mixed or Premixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
8. Seal the plate with a plate sealer, cover it with the lid. Wrap a rubber band around the plate holder, plate and lid and incubate with agitation on a plate shaker overnight at 4°C or 1 hour at room temperature (20-25°C). *An overnight incubation (16-18 hr) may improve assay sensitivity for some analytes.*

Add 200 μ L Assay Buffer per well



Shake 10 min, RT

Vacuum

- Add 25 μ L Standard or Control to appropriate wells
- Add 25 μ L Assay Buffer to background and sample wells
- Add 25 μ L Samples to sample wells
- Add 25 μ L Matrix to background, standards and control wells
- Add 25 μ L Beads to each well



Incubate overnight at 4°C or 1 hour at RT with shaking

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) if 1st incubation at Step 8 is overnight. If Step 8 is a 1 hour incubation, incubate at room temperature for 30 minutes. **DO NOT VACUUM AFTER INCUBATION.**
13. Add 25 μL Streptavidin-Phycoerythrin to each well containing the 25 μL of Detection Antibodies.
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 /well Wash Buffer, 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™ IS, 200™, or HTS.
19. Save and analyze the Median Fluorescent Intensity (MFI) data using a weighted 5-parameter logistic or spline curve-fitting method for calculating cytokine/chemokines concentrations in samples.



Vacuum and wash
2X with 200 μL
Wash Buffer

Add 25 μL Detection Antibody
per well



Incubate 1 hour
(overnight
protocol) or 30
minutes (1 hour
protocol) at RT

Do Not Vacuum

Add 25 μL Streptavidin-
Phycoerythrin per well



Incubate for 30
minutes at RT

Vacuum and wash
2X with 200 μL
Wash Buffer

Add 150 μL Sheath Fluid per
well

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

EQUIPMENT SETTINGS

These specifications are for the Luminex 100™ IS v.1.7 or Luminex 100™ IS v2.1/2.2, 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		50, per bead		50, per bead	
Sample Size:	100 µL		100 µL		100 µL		100 µL	
Gate Settings	8,000 to 15,000							
Time Out	60 seconds							
Bead Set:	14-Plex Premix Beads		26-Plex Premix Beads		39-Plex Premix Beads		Customizable 42-Plex Beads	
	GM-CSF	14	Eotaxin	4	EGF	2	EGF	2
	IFN γ	20	G-CSF	12	Eotaxin	4	Eotaxin	4
	IL-1 β	24	GM-CSF	14	FGF-2	6	FGF-2	6
	IL-2	28	IFN α 2	18	Flt-3 Ligand	8	Flt-3 Ligand	8
	IL-4	32	IFN γ	20	Fractalkine	10	Fractalkine	10
	IL-5	34	IL-1 α	22	G-CSF	12	G-CSF	12
	IL-6	36	IL-1 β	24	GM-CSF	14	GM-CSF	14
	IL-7	38	IL-2	28	GRO	16	GRO	16
	IL-8	40	IL-3	30	IFN α 2	18	IFN α 2	18
	IL-10	44	IL-4	32	IFN γ	20	IFN γ	20
	IL-12 (p70)	48	IL-5	34	IL-1 α	22	IL-1 α	22
	IL-13	50	IL-6	36	IL-1 β	24	IL-1 β	24
	MCP-1	58	IL-7	38	IL-1ra	26	IL-1ra	26
	TNF α	80	IL-8	40	IL-2	28	IL-2	28
			IL-10	44	IL-3	30	IL-3	30
			IL-12 (p40)	46	IL-4	32	IL-4	32
			IL-12 (p70)	48	IL-5	34	IL-5	34
			IL-13	50	IL-6	36	IL-6	36
			IL-15	52	IL-7	38	IL-7	38
			IL-17A	54	IL-8	40	IL-8	40
			IP-10	56	IL-9	42	IL-9	42
			MCP-1	58	IL-10	44	IL-10	44
			MIP-1 α	64	IL-12 (p40)	46	IL-12 (p40)	46
			MIP-1 β	66	IL-12 (p70)	48	IL-12 (p70)	48
			TNF α	80	IL-13	50	IL-13	50
			TNF β	82	IL-15	52	IL-15	52
					IL-17A	54	IL-17A	54
					IP-10	56	IP-10	56
					MCP-1	58	MCP-1	58
					MCP-3	60	MCP-3	60
					MDC	62	MDC	62
					MIP-1 α	64	MIP-1 α	64
					MIP-1 β	66	MIP-1 β	66
					sCD40L	74	PDGF-AA	68
					sIL-2R α	76	PDGF-AB/BB	70
					TGF α	78	RANTES	72
					TNF α	80	sCD40L	74
					TNF β	82	sIL-2R α	76
					VEGF	86	TGF α	78
							TNF α	80
							TNF β	82
							VEGF	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 MILLIPORE website www.millipore.com/techlibrary/index.do using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Standard Comparison

The following standards with known values of mass and standards received from the National Institute of Biological Standards and Controls (NIBSC) with assigned bioassay units and approximate mass determinations were assayed together to provide the following conversion factor:

Cytokine*	NIBSC Catalog #	NIBSC Version #	MILLIPLEX™ Human Cytokine Standard	Equivalence to NIBSC Reference Standard (IU/ng)
G-CSF	88/502	V04	2.5 ng/vial	106.61
GM-CSF	88/646	V03	2.5 ng/vial	9.53
IFN α 2	94/784	V04	2.5 ng/vial	515.46
IFN γ	88/606	V05	2.5 ng/vial	33.98
IL-1 α	86/632	V02	2.5 ng/vial	117.51
IL-1 β	86/680	V02	2.5 ng/vial	85.47
IL-2	86/504	V03	2.5 ng/vial	8.84
IL-3	91/510	V04	2.5 ng/vial	3.00
IL-4	88/656	V04	2.5 ng/vial	2.35
IL-5	90/586	V04	2.5 ng/vial	16.13
IL-6	89/548	V02	2.5 ng/vial	72.67
IL-7	90/530	V03	2.5 ng/vial	156.49
IL-8	89/520	V04	2.5 ng/vial	1.25
IL-9	91/678	V04	2.5 ng/vial	35.21
IL-10	93/722	V04	2.5 ng/vial	3.83
IL-12 (p70)	95/544	V04	2.5 ng/vial	9.76
IL-13	94/622	V03	2.5 ng/vial	0.22
IL-15	95/554	V03	2.5 ng/vial	14.86
MCP-1	92/794	V04	2.5 ng/vial	0.58
MIP-1 α	92/518	V04	2.5 ng/vial	0.17
RANTES	92/520	V03	2.5 ng/vial	8.82
IL-2R α	97/600	V04	2.5 ng/vial	171.82
TNF α	88/786	V05	2.5 ng/vial	99.57
TNF β	87/640	V04	2.5 ng/vial	224.22

*Cytokines/Chemokines listed in this table are the only ones currently available from NIBSC.

Cross-Reactivity

There was no or negligible cross-reactivity between the antibodies and any of the other analytes in this panel.

Assay Sensitivities (minimum detectable concentrations, pg/mL)

MinDC: Minimum Detectable Concentration is calculated by the StatLIA® Immunoassay Analysis Software from Brendan Technologies. It measures 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.

Cytokine	Overnight Protocol (N = 8 assays)		Short Protocol (N = 4 assays)	
	Mean MinDC	Mean MinDC + 2SD	Mean MinDC	Mean MinDC + 2SD
EGF	2.7	5.4	5.3	12.0
Eotaxin	1.2	2.2	2.1	5.2
FGF-2	1.8	3.2	6.0	13.4
Flt-3L	2.6	4.7	6.1	14.1
Fractalkine	6.0	10.6	7.6	16.7
G-CSF	0.5	0.9	3.9	10.1
GM-CSF	9.5	18.9	2.3	5.7
GRO	10.1	18.2	11.4	25.4
IFN α 2	24.5	40.6	7.2	16.5
IFN γ	0.1	0.3	0.4	0.8
IL-1 α	3.5	6.4	1.5	3.6
IL-1 β	0.4	0.7	0.7	1.6
IL-1ra	2.9	5.5	2.3	5.3
IL-2	0.3	0.6	0.4	1.0
IL-3	2.1	3.8	9.8	23.8
IL-4	0.6	1.1	0.6	1.7
IL-5	0.1	0.1	0.1	0.4
IL-6	0.3	0.7	0.4	0.9
IL-7	1.8	4.0	1.0	2.1
IL-8	0.2	0.3	0.3	0.7
IL-9	0.7	1.2	1.1	2.5
IL-10	0.3	0.5	0.3	0.8
IL-12(p40)	10.5	19.9	12.3	28.8
IL-12(p70)	0.4	0.8	0.9	2.3
IL-13	0.4	0.9	0.3	0.7
IL-15	0.4	0.7	0.6	1.3
IL-17A	0.2	0.3	0.4	1.1
IP-10	1.2	2.2	1.3	3.0
MCP-1	0.9	1.6	1.2	2.8
MCP-3	2.0	3.7	5.2	11.8
MDC	3.7	6.9	2.4	5.4
MIP-1 α	3.5	6.4	6.6	14.9
MIP-1 β	4.5	8.9	3.2	7.1
sCD40L	4.9	9.0	5.2	12.3
sIL-2R α	4.4	7.7	7.5	17.1
TGF α	0.4	0.8	1.4	3.1
TNF α	0.1	0.1	0.2	0.6
TNF β	1.9	3.4	4.1	9.9
VEGF	5.8	10.3	10.1	22.7
PDGF-AA	0.1	0.3	0.3	0.6
PDGF-AB/BB	7.3	15.8	12.2	28.2
RANTES	1.0	2.0	1.6	3.7

Precision

Intra-assay precision is generated from the mean of the %CV's from eight 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 four different experiments.

Cytokine	Intra-Assay %CV	Inter-Assay %CV
EGF	7.8	15.2
Eotaxin	7.4	15.0
FGF-2	7.5	12.8
Flt-3L	6.0	8.9
Fractalkine	5.3	10.1
G-CSF	6.6	6.4
GM-CSF	10.4	12.6
GRO	5.1	11.6
IFNα2	11.4	10.1
IFNγ	4.6	5.8
IL-1α	8.2	16.8
IL-1β	6.1	7.0
IL-1ra	4.6	6.0
IL-2	6.4	7.4
IL-3	13.8	17.2
IL-4	5.0	3.7
IL-5	7.3	13.0
IL-6	8.1	11.6
IL-7	4.9	8.3
IL-8	7.1	11.6
IL-9	11.0	13.2
IL-10	5.2	9.5
IL-12(p40)	7.2	12.7
IL-12(p70)	8.7	14.3
IL-13	4.8	8.9
IL-15	6.7	9.5
IL-17A	4.5	9.9
IP-10	4.7	11.2
MCP-1	6.1	12.0
MCP-3	7.1	13.1
MDC	5.1	9.3
MIP-1α	5.7	14.4
MIP-1β	5.3	10.6
sCD40L	8.0	7.5
sIL-2Rα	6.4	7.3
TGFα	12.0	9.7
TNFα	10.5	15.9
TNFβ	8.3	4.3
VEGF	5.8	7.9
PDGF-AA	8.1	12.7
PDGF-AB/BB	11.5	10.9
RANTES	6.2	15.7

Accuracy

Spike Recovery: The data represent mean percent recovery of six levels of spiked standards ranging from 3 to 10,000 pg/mL in serum matrices in eight independent experiments.

Cytokine	Spike Recovery in Serum Matrix (6 Point Spikes)
EGF	111.2
Eotaxin	102.1
FGF-2	100.7
Flt-3L	101.0
Fractalkine	102.9
G-CSF	100.9
GM-CSF	110.6
GRO	118.3
IFN α 2	116.6
IFN γ	99.2
IL-1 α	103.0
IL-1 β	100.1
IL-1ra	101.5
IL-2	100.5
IL-3	97.8
IL-4	99.8
IL-5	103.7
IL-6	100.0
IL-7	99.8
IL-8	101.9
IL-9	100.8
IL-10	99.7
IL-12(p40)	124.2
IL-12(p70)	100.4
IL-13	100.2
IL-15	100.5
IL-17A	97.2
IP-10	99.0
MCP-1	100.7
MCP-3	102.7
MDC	108.2
MIP-1 α	102.8
MIP-1 β	99.3
sCD40L	100.7
sIL-2R α	102.6
TGF α	101.3
TNF α	98.7
TNF β	100.8
VEGF	107.7
PDGF-AA	104.1
PDGF-AB/BB	83.9
RANTES	89.5

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Filter plate will not vacuum	Vacuum pressure is insufficient Samples have insoluble particles Sample too viscous	Increase vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds. Centrifuge samples just prior to assay set-up and use supernatant. If high lipid concentration, after centrifugation, remove lipid layer and use supernatant. May need to dilute sample.
Insufficient bead count	Vacuum pressure too high Bead mix prepared incorrectly Samples cause interference due to particulate matter or viscosity Probe height not adjusted correctly	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds. Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting into the plate. See above. Also sample probe may need to be cleaned with alcohol flush, backflush and washes; or, if needed, probe should be removed and sonicated. Adjust probe to 3 alignment discs in well H6.
Plate leaked	Vacuum pressure too high Plate set directly on table or absorbent towels during incubations or reagent additions Insufficient blotting of filter plate bottom causing wicking Pipette touching plate filter during additions Probe height not adjusted correctly	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (prewetted) plate and continue. Set plate on plate stand or raised edge so bottom of filter is not touching any surface. Blot the bottom of the filter plate well with absorbent towels after each wash step. Pipette to the side of well. Adjust probe to 3 alignment discs in well H6.
Background is too high	Background wells were contaminated Matrix used has endogenous analyte or interference Insufficient washes	Avoid cross-well contamination by using sealer appropriately and by pipeting with multichannel pipets without touching reagent in plate. Check matrix ingredients for crossreacting components (e.g. interleukin modified tissue culture medium). Increase number of washes.

<p>Beads not in region or gate</p>	<p>Luminex not calibrated correctly or recently</p> <p>Gate settings not adjusted correctly</p> <p>Wrong bead regions in protocol template</p> <p>Incorrect sample type used</p> <p>Instrument not washed or primed</p> <p>Beads were exposed to light</p>	<p>Calibrate Luminex based on instrument manufacturer's instructions at least once a week or if temperature has changed by >3°C.</p> <p>Some Luminex instruments (e.g. Bio-Plex) require different gate settings than those described in the kit protocol. Use instrument default settings.</p> <p>Check kit protocol for correct bead regions or analyte selection.</p> <p>Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required.</p> <p>Prime the Luminex 4 times to eliminate air bubbles. Wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid.</p> <p>Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.</p>
<p>Signal for whole plate is same as background</p>	<p>Incorrect or no Detection Antibody was added</p> <p>Streptavidin-Phycoerythrin was not added</p>	<p>Add appropriate Detection Antibody and continue.</p> <p>Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been vacuumed out, sensitivity may be low.</p>
<p>Low signal for standard curve</p>	<p>Detection Antibody may have been vacuumed out prior to adding Streptavidin Phycoerythrin</p> <p>Incubations done at incorrect temperatures, timings or agitation</p>	<p>May need to repeat assay if desired sensitivity not achieved.</p> <p>Assay conditions need to be checked.</p>
<p>Signals too high, standard curves are saturated</p>	<p>Calibration target value set too high</p> <p>Plate incubation was too long with standard curve and samples</p>	<p>With some Luminex instruments (e.g. Bio-Plex) default target setting for RP1 calibrator is set at High PMT. Use low target value for calibration and reanalyze plate.</p> <p>Use shorter incubation time.</p>
<p>Sample readings are out of range</p>	<p>Samples contain no or below detectable levels of analyte</p> <p>Samples contain analyte concentrations higher than highest standard point</p> <p>Standard curve was saturated at higher end of curve</p>	<p>If below detectable levels, it may be possible to use higher sample volume. Check with tech support for appropriate protocol modifications.</p> <p>Samples may require dilution and reanalysis for that particular analyte.</p> <p>See above.</p>

<p>High variation in samples and/or standards</p>	<p>Multichannel pipet may not be calibrated</p> <p>Plate washing was not uniform</p> <p>Samples may have high particulate matter or other interfering substances</p> <p>Plate agitation was insufficient</p> <p>Cross-well contamination</p>	<p>Calibrate pipets.</p> <p>Confirm all reagents are vacuumed out completely in all wash steps.</p> <p>See above.</p> <p>Plate should be agitated during all incubation steps using a vertical plate shaker at a speed where beads are in constant motion without splashing.</p> <p>Check when reusing plate sealer that no reagent has touched sealer.</p> <p>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.</p>
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REPLACEMENT REAGENTS

Human Cytokine Standard
 Human Cytokine Quality Controls
 Serum Matrix
 Human Cytokine Detection Antibodies

Catalog #
 MXH8060
 MXH8060-2
 MXH6060
 MXH6060-2
 MXHSM (optional)
 MXH1060-1
 MXH1060-2
 MXH1060-3
 MXH1060-4

Streptavidin-Phycoerythrin
Use with Cat. # MXH1060-1
Use with Cat. # MXH1060-2
Use with Cat. # MXH1060-3
Use with Cat. # MXH1060-4

L-SAPE9
 L-SAPE3
 L-SAPE10
 L-SAPE11
 L-AB
 LBD
 MX-PLATE
 L-WB

Assay Buffer
 Bead Diluent
 Set of two 96-Well Filter Plates with Sealers
 10X Wash Buffer
Antibody-Immobilized Beads

<u>Cytokine</u>	<u>Bead #</u>	<u>Cat. #</u>	<u>Cytokine</u>	<u>Bead #</u>	<u>Cat. #</u>
EGF	2	MXHEGF	IL-12(040)	46	MXH12P40
Eotaxin	4	MXHETXN	IL-12(p70)	48	MXH12P70
FGF-2	6	MXHFGF2	IL-13	50	MXHIL-13
Flt-3 Ligand	8	MXHFLT3L	IL-15	52	MXHIL-15
Fractalkine	10	MXHFKN	IL-17A	54	MXHIL-17A
G-CSF	12	MXHGCSF	IP-10	56	MXHIP10
GM-CSF	14	MXHGMCSF	MCP-1	58	MXHMCP-1
GRO	16	MXHGRO	MCP-3	60	MXHMCP3
IFN α 2	18	MXHIFNA2	MDC	62	MXHMDC
IFN γ	20	MXHIFNG	MIP-1 α	64	MXHMIP-1A
IL-1 α	22	MXHIL-1A	MIP-1 β	66	MXHMIP-1B
IL-1 β	24	MXHIL-1B	PDGF-AA	68	MXHPDGFAA
IL-1ra	26	MXHIL-1RA	PDGF-AB/BB	70	MXHPDGFAF-BB
IL-2	28	MXHIL-2	RANTES	72	MXHRNTS
sIL-2 α	76	MXHIL-2RA	sCD40L	74	MXHCD40L
IL-3	30	MXHIL-3	TGF α	78	MXHTGF-A
IL-4	32	MXHIL-4	TNF- α	80	MXHTNF-A
IL-5	34	MXHIL-5	TNF β	82	MXHTNF-B
IL-6	36	MXHIL-6	VEGF	86	MXHVEGF
IL-7	38	MXHIL-7	Premixed 14-plex Beads		MXHPMX14
IL-8	40	MXHIL-8	Premixed 26-plex Beads		MXHPMX26
IL-9	42	MXHIL-9	Premixed 39-plex Beads		MXHPMX39
IL-10	44	MXHIL-10	Premixed 42-plex Beads		MXHPMX39 + MXHRNTS, MXHPDGFAA, MXHPDGFAF

ORDERING INFORMATION

To place an order:

To assure the clarity of your custom cytokine 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[®] Cytokine Analytes/Serum Matrix Requirements

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Material Safety Data Sheets for Millipore products may be ordered by fax or phone or through our website at www.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)	400 pg/mL Standard	QC-2 Control									
B	0 pg/mL Standard (Background)	400 pg/mL Standard	QC-2 Control									
C	3.2 pg/mL Standard	2000 pg/mL Standard	Sample 1									
D	3.2 pg/mL Standard	2000 pg/mL Standard	Sample 1									
E	16 pg/mL Standard	10,000 pg/mL Standard	Sample 2									
F	16 pg/mL Standard	10,000 pg/mL Standard	Sample 2									
G	80 pg/mL Standard	QC-1 Control	Etc.									
H	80 pg/mL Standard	QC-1 Control										