MILLIPLEX® MAP

MOUSE CYTOKINE / CHEMOKINE KIT PROTOCOL
96 Well Plate Assay

#MPXMCYTO-70K or
#MPXMCYTO70KPMX13 (premixed) or
#MPXMCYTO70KPMX22 (premixed) or
#MPXMCYTO70KPMX32 (premixed)

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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 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™ Mouse 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™ Mouse 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 (13-, 22- or 32-plex).
  - Choose any combination of analytes from our panel of 32 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™ Mouse Cytokine / Chemokine kit is to be used for the simultaneous quantification of the following 32 mouse cytokines and chemokines: Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, 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.

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
Mouse Cytokine / Chemokine Standard	MXM8070 or MXM8070-2	lyophilized	1 vial
Mouse Cytokine Quality Controls 1 and 2	MXM6070 or MXM6070-2	lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	MXMSM	lyophilized	1 vial
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
Mouse Cytokine Detection Antibodies	MXM1070-1 or MXM1070-2 or MXM1070-3	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE3 *(Use with Cat. # MXM1070-1)* or L-SAPE4 *(Use with Cat. # MXM1070-2)* or L-SAPE10 *(Use with Cat. # MXM1070-3)*	3.2 mL	1 bottle
Mixing Bottle (not provided with premixed panel)	----------	----------	1 bottle

Mouse Cytokine / Chemokine Antibody-Immobilized Premixed Beads:

Premixed 13-plex Beads	MXMPMX13	3.5 mL	1 bottle
Premixed 22-plex Beads	MXMPMX22	3.5 mL	1 bottle
Premixed 32-plex Beads	MXMPMX32	3.5 mL	1 bottle

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

Mouse Cytokine / Chemokine Antibody-Immobilized Beads:

Bead/Analyte Name	Luminex Bead Region	Customizable 32 Analytes (50X concentration, 90µL) Available	Cat. #	13-Plex Premixed Beads	22-Plex Premixed Beads	32-Plex Premixed Beads
Anti-Mouse Eotaxin Bead	1	✓	MXMETXN			✓
Anti-Mouse G-CSF Bead	3	✓	MXMGCSF		✓	✓
Anti-Mouse GM-CSF Bead	5	✓	MXMGMCSF	✓	✓	✓
Anti-Mouse IFNγ Bead	7	✓	MXMIFNG	✓	✓	✓
Anti-Mouse IL-1α Bead	9	✓	MXMIL-1A		✓	✓
Anti-Mouse IL-1β Bead	11	✓	MXMIL-1B	✓	✓	✓
Anti-Mouse IL-2 Bead	13	✓	MXMIL-2	✓	✓	✓
Anti-Mouse IL-3 Bead	15	✓	MXMIL-3			✓
Anti-Mouse IL-4 Bead	17	✓	MXMIL-4	✓	✓	✓
Anti-Mouse IL-5 Bead	19	✓	MXMIL-5	✓	✓	✓
Anti-Mouse IL-6 Bead	21	✓	MXMIL-6	✓	✓	✓
Anti-Mouse IL-7 Bead	23	✓	MXMIL-7	✓	✓	✓
Anti-Mouse IL-9 Bead	66	✓	MXMIL-9		✓	✓
Anti-Mouse IL-10 Bead	27	✓	MXMIL-10	✓	✓	✓
Anti-Mouse IL-12 (p40) Bead	29	✓	MXM12P40			✓
Anti-Mouse IL-12 (p70) Bead	62	✓	MXM12P70	✓	✓	✓
Anti-Mouse IL-13 Bead	33	✓	MXMIL-13	✓	✓	✓
Anti-Mouse IL-15 Bead	35	✓	MXMIL-15		✓	✓
Anti-Mouse IL-17 Bead	37	✓	MXMIL-17		✓	✓
Anti-Mouse IP-10 Bead	39	✓	MXMIP10		✓	✓
Anti-Mouse KC Bead	42	✓	MXMKC		✓	✓
Anti-Mouse LIF Bead	44	✓	MXMLIF			✓
Anti-Mouse LIX Bead	45	✓	MXMLIX			✓
Anti-Mouse MCP-1 Bead	49	✓	MXMMCP-1	✓	✓	✓
Anti-Mouse M-CSF Bead	10	✓	MXMMCSF			✓
Anti-Mouse MIG Bead	57	✓	MXMMIG			✓
Anti-Mouse MIP-1α Bead	53	✓	MXMMIP-1A		✓	✓
Anti-Mouse MIP-1β Bead	55	✓	MXMMIP-1B			✓
Anti-Mouse MIP-2 Bead	40	✓	MXMMIP-2			✓
Anti-Mouse RANTES Bead	59	✓	MXMRNTS		✓	✓
Anti-Mouse TNFα Bead	61	✓	MXMTNF-A	✓	✓	✓
Anti-Mouse VEGF Bead	63	✓	MXMVEGF			✓

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 (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 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 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 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 and control wells. If samples are diluted in Assay Buffer, use the Assay Buffer as matrix.
- For serum/plasma samples that require further dilution beyond 1:1, use the Serum Matrix provided in the kit as the 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 and no 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.
- Serum samples should be diluted 1:1 in the Assay Buffer provided in the kit (i.e. one part serum sample into one part Assay Buffer). When further dilution beyond 1:1 is required, use Serum Matrix as the 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.
- Plasma samples should be diluted 1:1 in the Assay Buffer provided in the kit (i.e. one part plasma sample into one part Assay Buffer). When further dilution beyond 1:1 is required, use Serum Matrix as the 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.

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.

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 Assay Buffer. 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 Assay Buffer.

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 Assay Buffer.

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 ≤ -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 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 2.0 mL Assay Buffer 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 Mouse Cytokine Standard

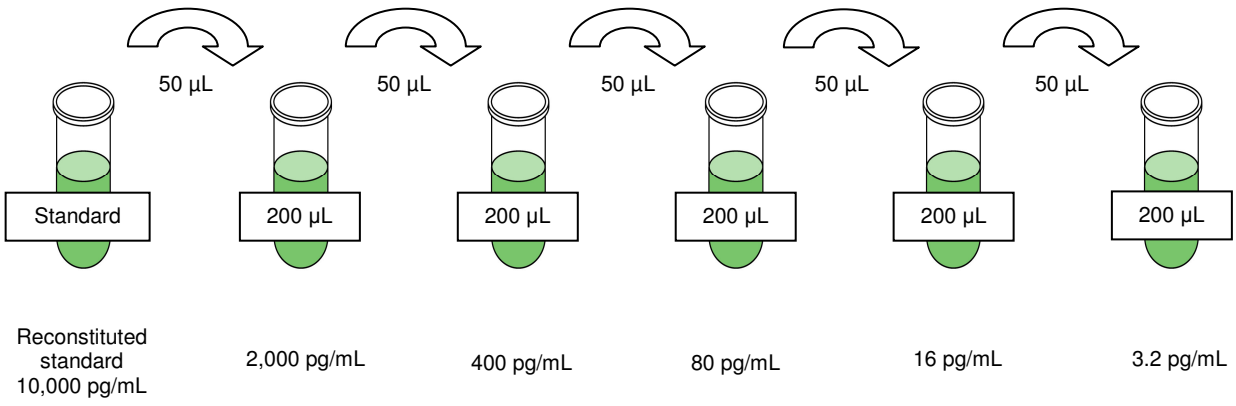
1.) Prior to use, reconstitute the Mouse Cytokine Standard with 250 µL deionized water to give a 10,000 pg/mL concentration of standard for all analytes. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes and then transfer the standard to an appropriately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

2). Preparation of Working Standards

Label five polypropylene microfuge tubes 2,000, 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 2,000 pg/mL tube, mix well and transfer 50 µL of the 2,000 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
2,000	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, 2,000, 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 µL of Wash Buffer into each well of the Microtiter Filter Plate. Seal and shake on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Remove Wash Buffer by vacuum. **(NOTE: DO NOT INVERT PLATE.)** Blot excess Wash 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 pg/mL 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, use proper control culture medium as the matrix solution.
6. Add 25 µL of Sample (diluted one part serum or plasma to one part Assay Buffer) into the appropriate wells.
7. Vortex Mixing Bottle and add 25 µL 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 2 hours at room temperature (20-25°C). *An overnight incubation (16-18 hr) may improve assay sensitivity for some analytes.*

Add 200 µL Wash Buffer per well

↓ Shake 10 min, RT

↓ Vacuum

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

↓ Incubate overnight at 4°C or 2 hours 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 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.
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™, HTS.
19. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating cytokine/chemokines concentrations in samples. (Note: For diluted samples, multiply the calculated concentration by the dilution factor.)

Vacuum and wash 2X with 200 µL Wash Buffer

Add 25 µL Detection Antibody per well

Incubate 1 hour 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:	13-Plex Premix Beads		22-Plex Premix Beads		32-Plex Premix Beads		Customizable 32-Plex Beads	
	GM-CSF	5	G-CSF	3	Eotaxin	1	Eotaxin	1
	IFNγ	7	GM-CSF	5	G-CSF	3	G-CSF	3
	IL-1β	11	IFNγ	7	GM-CSF	5	GM-CSF	5
	IL-2	13	IL-1α	9	IFNγ	7	IFNγ	7
	IL-4	17	IL-1β	11	IL-1α	9	IL-1α	9
	IL-5	19	IL-2	13	IL-1β	11	IL-1β	11
	IL-6	21	IL-4	17	IL-2	13	IL-2	13
	IL-7	23	IL-5	19	IL-3	15	IL-3	15
	IL-10	27	IL-6	21	IL-4	17	IL-4	17
	IL-12 (p70)	62	IL-7	23	IL-5	19	IL-5	19
	IL-13	33	IL-9	66	IL-6	21	IL-6	21
	MCP-1	49	IL-10	27	IL-7	23	IL-7	23
	TNFα	61	IL-12 (p70)	62	IL-9	66	IL-9	66
			IL-13	33	IL-10	27	IL-10	27
			IL-15	35	IL-12 (p40)	29	IL-12 (p40)	29
			IL-17	37	IL-12 (p70)	62	IL-12 (p70)	62
			IP-10	39	IL-13	33	IL-13	33
			KC	42	IL-15	35	IL-15	35
			MCP-1	49	IL-17	37	IL-17	37
			MIP-1α	53	IP-10	39	IP-10	39
			RANTES	59	KC	42	KC	42
			TNFα	61	LIF	44	LIF	44
					LIX	45	LIX	45
					MCP-1	49	MCP-1	49
					M-CSF	10	M-CSF	10
					MIG	57	MIG	57
					MIP-1α	53	MIP-1α	53
					MIP-1β	55	MIP-1β	55
					MIP-2	40	MIP-2	40
					RANTES	59	RANTES	59
					TNFα	61	TNFα	61
					VEGF	63	VEGF	63

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

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 = 6 Assays)			2 Hour Protocol
	MinDC	MinDC+2SD	LLOQ (est.)*	MinDC
Eotaxin	4.4	5.8	6.0	5.5
G-CSF	0.9	1.6	1.0	2.8
GM-CSF	5.6	6.8	10.0	9.5
IFNγ	0.9	1.5	1.0	2.3
IL-1α	5.1	11.8	8.0	5.7
IL-1β	2.0	3.5	2.0	2.7
IL-2	0.8	1.1	1.0	0.8
IL-3	0.7	1.1	1.0	1.8
IL-4	0.4	0.5	1.0	0.5
IL-5	0.7	1.1	1.0	1.2
IL-6	1.8	4.0	4.0	3.4
IL-7	0.9	1.3	1.3	2.9
IL-9	6.0	8.0	10.0	11.5
IL-10	3.3	5.8	1.5	8.0
IL-12 (p40)	4.9	7.9	8.0	10.2
IL-12 (p70)	4.1	6.2	6.0	5.7
IL-13	6.3	9.8	25.0	10.8
IL-15	6.5	11.7	8.0	9.8
IL-17	0.5	0.6	1.0	0.8
IP-10	0.6	0.8	1.0	1.1
KC	1.4	2.2	1.0	1.8
LIF	0.8	1.1	1.0	1.9
LIX	7.6	13.9	8.0	11.2
MCP-1	5.3	7.5	5.0	9.1
M-CSF	1.1	1.9	2.0	1.8
MIG	1.0	2.3	1.0	1.9
MIP-1α	8.7	10.4	12.0	29.5
MIP-1β	10.1	13.5	12.0	14.8
MIP-2	63.6	105.5	125.0	54.9
RANTES	2.5	3.5	5.0	1.9
TNFα	1.0	1.5	1.2	1.4
VEGF	0.3	0.4	1.0	0.4

*LLOQ=Lowest Level of Quantification

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 4 - 8 reportable results across two different concentrations of cytokine in 4 different experiments.

Cytokine	Overnight Protocol		2 Hour Protocol
	Intra-assay %CV	Inter-assay %CV	Intra-assay %CV
Eotaxin	7.4	6.8	5.2
G-CSF	8.4	8.0	6.6
GM-CSF	8.0	5.4	4.7
IFNγ	5.9	7.1	5.2
IL-1α	7.3	6.2	5.7
IL-1β	8.1	7.0	6.5
IL-2	5.6	4.2	4.2
IL-3	9.1	5.4	5.0
IL-4	8.1	8.5	5.3
IL-5	9.7	4.7	7.0
IL-6	10.4	7.9	7.8
IL-7	8.8	6.9	7.7
IL-9	8.0	7.1	5.6
IL-10	7.0	10.1	3.8
IL-12 (p40)	8.7	9.0	5.2
IL-12 (p70)	7.6	5.5	4.8
IL-13	11.9	10.9	10.5
IL-15	9.4	11.9	9.1
IL-17	7.5	6.3	4.0
IP-10	10.0	10.3	6.9
KC	9.9	10.9	7.5
LIF	9.2	7.6	6.0
LIX	5.2	5.9	6.4
MCP-1	5.8	4.4	3.0
M-CSF	11.3	11.5	6.0
MIG	10.3	20.4	17.5
MIP-1α	7.0	8.4	6.8
MIP-1β	5.0	6.3	4.7
MIP-2	21.3	21.2	22.6
RANTES	10.3	13.0	6.8
TNFα	16.3	11.8	11.5
VEGF	7.9	12.1	5.6

Accuracy

Spike Recovery: The data represent mean percent recovery of six levels of spiked standards in serum matrices.

Cytokine	Overnight Protocol	2 Hour Protocol
	% Recovery in Serum Matrix	% Recovery in Serum Matrix
Eotaxin	101.1	100.7
G-CSF	100.1	100.9
GM-CSF	99.2	105.6
IFNγ	99.1	101.1
IL-1α	98.4	103.6
IL-1β	101.4	101.1
IL-2	100.3	100.1
IL-3	100.3	100.3
IL-4	100.6	100.2
IL-5	100.3	100.2
IL-6	100.4	103.5
IL-7	100.1	100.6
IL-9	101.5	98.6
IL-10	100.4	102.6
IL-12 (p40)	102.5	108.6
IL-12 (p70)	100.4	101.0
IL-13	100.1	107.2
IL-15	103.8	105.0
IL-17	99.6	100.4
IP-10	103.2	100.1
KC	100.5	101.4
LIF	100.2	100.2
LIX	100.5	139.8
MCP-1	101.7	100.0
M-CSF	100.4	100.8
MIG	100.8	99.2
MIP-1α	100.4	85.3
MIP-1β	103.0	142.1
MIP-2	123.2	79.6
RANTES	100.5	109.4
TNFα	105.1	100.6
VEGF	100.1	100.3

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
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 particles	Centrifuge samples just prior to assay set-up and use supernatant. If high lipid concentration, after centrifugation, remove lipid layer and use 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 incorrectly	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.
	Samples cause interference due to particulate matter or viscosity	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.
	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 0.2mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (prewetted) plate and continue.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate stand 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 well.
	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 by pipeting with multichannel pipets without touching reagent in plate.
	Matrix used has endogenous analyte or interference	Check matrix ingredients for crossreacting 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°C.
	Gate settings not adjusted correctly	Some Luminex instruments (e.g. Bio-Plex) 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 eliminate 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 incorrect 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 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.
	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 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 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 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	**Catalog #**
Mouse Cytokine Standard	MXM8070 MXM8070-2
Mouse Cytokine Quality Controls	MXM6070 MXM6070-2
Serum Matrix	MXMSM
Mouse Cytokine Detection Antibodies	MXM1070-1 MXM1070-2 MXM1070-3
Streptavidin-Phycoerythrin	
Use with Cat. # MXM1070-1	L-SAPE3
Use with Cat. # MXM1070-2	L-SAPE4
Use with Cat. # MXM1070-3	L-SAPE10
Assay Buffer	L-AB
Set of two 96-Well Filter Plates with Sealers	MX-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Beads

Cytokine	*Bead #*	*Cat. #*
Eotaxin	*1*	*MXMETXN*
G-CSF	*3*	*MXMGCSF*
GM-CSF	*5*	*MXMGMCSF*
IFNγ	*7*	*MXMIFNG*
IL-1α	*9*	*MXMIL-1A*
IL-1β	*11*	*MXMIL-1B*
IL-2	*13*	*MXMIL-2*
IL-3	*15*	*MXMIL-3*
IL-4	*17*	*MXMIL-4*
IL-5	*19*	*MXMIL-5*
IL-6	*21*	*MXMIL-6*
IL-7	*23*	*MXMIL-7*
IL-9	*66*	*MXMIL-9*
IL-10	*27*	*MXMIL-10*
IL-12 (p40)	*29*	*MXM12P40*
IL-12 (p70)	*62*	*MXM12P70*
IL-13	*33*	*MXMIL-13*
IL-15	*35*	*MXMIL-15*

Cytokine	Bead #	Cat. #
IL-17	*37*	*MXMIL-17*
IP-10	*39*	*MXMIP10*
KC	*42*	*MXMKC*
LIF	*44*	*MXMLIF*
LIX	*45*	*MXMLIX*
MCP-1	*49*	*MXMMCP-1*
M-CSF	*10*	*MXMMCSF*
MIG	*57*	*MXMMIG*
MIP-1α	*53*	*MXMMIP-1A*
MIP-1β	*55*	*MXMMIP-1B*
MIP-2	*40*	*MXMMIP-2*
RANTES	*59*	*MXMRNTS*
TNFα	*61*	*MXMTNF-A*
VEGF	*63*	*MXMVEGF*
Premixed 13-plex Beads		*MXMPMX13*
Premixed 22-plex Beads		*MXMPMX22*
Premixed 32-plex Beads		*MXMPMX32*

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- Quantity of kits
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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	2,000 pg/mL Standard										
D	3.2 pg/mL Standard	2,000 pg/mL Standard										
E	16 pg/mL Standard	10,000 pg/mL Standard										
F	16 pg/mL Standard	10,000 pg/mL Standard										
G	80 pg/mL Standard	QC-1 Control										
H	80 pg/mL Standard	QC-1 Control										