Rat Cytokine / Chemokine Magnetic Bead

96-Well Plate Assay

Cat. # RCYTOMAG-80K or
RCYTOMAG-80K-PMX

MILLIPLEX® MAP

RAT CYTOKINE / CHEMOKINE MAGNETIC BEAD PANEL KIT
96 Well Plate Assay

RCYTOMAG-80K or
RCYTOMAG-80K-PMX (premixed)

TABLE OF CONTENTS **PAGE**

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES

By purchasing this product, which contains fluorescently labeled microsphere beads authorized by Luminex Corporation ("Luminex"), you, the customer, acquire the right under Luminex's patent rights, if any, to use this product or any portion of this product, including without limitation the microsphere beads contained herein, only with Luminex's laser based fluorescent analytical test instrumentation marketed under the name of Luminex 100™ IS, 200™, HTS, FLEXMAP 3D™ MAGPIX®..

Rat Cytokine/Chemokine Magnetic Bead Panel

"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 direct interactions between cells and regulate processes taking place in the extracellular environment. Cytokines differ from hormones in that they act on a wider spectrum of target cells. Also, unlike hormones, they are not produced by specialized cells which are organized in specialized glands. The cytokine 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 the immune system and its multi-faceted response to most antigens, as well as disease states such as inflammatory disease, allergic reactions, irritable bowel disease (IBD), sepsis, and cancer.

To identify specific cytokines involved in any inflammatory or immune response, it might be necessary to screen panels of cytokines, often requiring some level of automation and/or high throughput. Magnetic Beads can make the process of automation and high throughput screening easier with features such as walk-away washing. Advantages even outside automation include:

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

Therefore, the **MILLIPLEX® *MAP*** Rat 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 in a **magnetic bead** format, 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® *MAP* Rat Cytokine / Chemokine panel is the most versatile system available for cytokine and chemokine research.

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

Millipore's MILLIPLEX® *MAP* Rat Cytokine / Chemokine kit is to be used for the simultaneous quantification of the following 23 rat cytokines and chemokines: Eotaxin, G-CSF, GM-CSF, GRO/KC, IFNγ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IL-17, IL-18, IP-10, Leptin, MCP-1, MIP-1α, RANTES, TNFα and VEGF.

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 magnetic beads known as MagPlex™-C microspheres.

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

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

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- 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 Antibody, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

Reagents Supplied	Catalog Number	Volume	Quantity
Rat Cytokine / Chemokine Standard	LRC-8080	lyophilized	1 vial
Rat Cytokine Quality Controls 1 and 2	LRC-6080	lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	LMC-SD	lyophilized	1 vial (required for serum and plasma samples only)
Bead Diluent (not provided with premixed panel)	LA-BD	4.0 mL	1 vial
Set of one 96-Well Plate with 2 sealers	----------	----------	1 plate 2 sealers
Assay Buffer	L-MAB	30 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	2 bottles
Rat Cytokine Detection Antibodies	LRC-1080-1 or LRC-1080-2 or LRC-1080-3	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE6 *(Use with Cat. #* LRC-1080-1 or LRC-1080-2*)* or L-SAPE4 *(Use with Cat. #* LRC-1080-3*)*	3.2 mL	1 bottle
Mixing Bottle (not provided with premixed panel)	----------	----------	1 bottle

Rat Cytokine / Chemokine Antibody-Immobilized Premixed Magnetic Beads:

Premixed 23-plex Beads	RCYPMX23-MAG	3.5 mL	1 bottle

Included Rat Cytokine / Chemokine Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel (see next page).

Rat Cytokine / Chemokine Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable 23 Analytes (50X concentration, 90µL) Available	Cat. #	23-Plex Premixed Beads
Anti-Rat G-CSF Bead	13	✓	MGCSF-MAG	✓
Anti-Rat Eotaxin Bead	14	✓	HETXN-MAG	✓
Anti-Rat GM-CSF Bead	18	✓	RGMCSF-MAG	✓
Anti-Rat IL-1α Bead	19	✓	RIL1A-MAG	✓
Anti-Rat Leptin Bead	22	✓	RCYLPTN-MAG	✓
Anti-Rat MIP-1α Bead	33	✓	RMIP1A-MAG	✓
Anti-Rat IL-4 Bead	34	✓	RIL4-MAG	✓
Anti-Rat IL-1β Bead	35	✓	RCYIL1B-MAG	✓
Anti-Rat IL-2 Bead	36	✓	RIL2-MAG	✓
Anti-Rat IL-6 Bead	37	✓	RCYIL6-MAG	✓
Anti-Rat IL-13 Bead	39	✓	RIL13-MAG	✓
Anti-Rat IL-10 Bead	51	✓	RIL10-MAG	✓
Anti-Rat IL-12p70 Bead	52	✓	RIL12P70-MAG	✓
Anti-Rat IFNγ Bead	53	✓	RIFNG-MAG	✓
Anti-Rat IL-5 Bead	55	✓	HIL5-MAG	✓
Anti-Rat IL-17 Bead	56	✓	MIL17-MAG	✓
Anti-Rat IL-18 Bead	57	✓	RIL18-MAG	✓
Anti-Rat MCP-1 Bead	62	✓	MCYMCP1-MAG	✓
Anti-Rat IP-10 Bead	65	✓	HIP10-MAG	✓
Anti-Rat GRO/KC Bead	72	✓	RGR0KC-MAG	✓
Anti-Rat VEGF Bead	73	✓	RCYVEGF-MAG	✓
Anti-Rat TNFα Bead	77	✓	MCYTNFA-MAG	✓
Anti-Rat RANTES Bead	78	✓	RRNTS-MAG	✓

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

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

Instrumentation / Materials

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

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

SAFETY PRECAUTIONS

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

TECHNICAL GUIDELINES

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

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.

- Do not mix or substitute reagents with those from other lots or sources.

- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- 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 ≤ -20°C for 1 month and at ≤ -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.
- When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 4 alignment discs. When reading the assay on FLEXMAP 3D™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc. When reading the assay on MAGPIX, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 2 alignment disc.
- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells. If samples are diluted in assay buffer, use the assay buffer as matrix.
- For serum/plasma samples that require further dilution beyond 1:5, 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 ≤ -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.
- It is recommended to centrifuge samples again at 3000xg for five minutes prior to assay setup.
- **Rat Serum should be diluted five-fold using the Serum Matrix as the sample diluent.** Dilute 1 part rat serum with 4 parts Serum Matrix (e.g. add 12 µL rat serum to 48 µL of Serum Matrix for duplicate samples). Alternatively, at step at Step 6 of the Immunoassay Procedure, add 20 µL of the Serum Matrix to each sample well followed by 5 µL undiluted serum sample added directly into the sample wells. **In some serum samples RANTES and GRO/KC may have high concentrations and may require further dilution (e.g. 1:20).**

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.
- It is recommended to centrifuge samples again at 3000xg for five minutes prior to assay setup.
- **Rat Plasma should be diluted five-fold using the Serum Matrix as the sample diluent.** Dilute 1 part rat plasma with 4 parts Serum Matrix (e.g. add 12 µL rat plasma to 48 µL of Serum Matrix for duplicate samples). Alternatively, at step at Step 6 of the Immunoassay Procedure, add 20 µL of the Serum Matrix to each sample well followed by 5 µL undiluted plasma sample added directly into the sample wells. **In some plasma samples RANTES and GRO/KC may have high concentrations and may require further dilution (e.g. 1:20).**

C. Preparation of Tissue Culture Supernatant:

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

NOTE:

- A maximum of 25 µL per well of 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 anti-coagulant 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 Bead Diluent. Vortex the mixed beads well. Unused portion may be stored at 2-8°C for up to one month. (Note: Due to the composition of magnetic beads, you may notice a slight color in the bead solution. This does not affect the performance of the beads or the kit.)

Example 1: When using 20 cytokine antibody-immobilized beads, add 60 µL from each of the 20 bead vials to the Mixing Bottle. Then add 1.8 mL Bead Diluent.

Example 2: When using 9 cytokine antibody-immobilized beads, add 60 µL from each of the 9 bead vials 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 ≤ -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 1.0 mL deionized water and 4.0 mL Assay Buffer to the bottle containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution. Leftover reconstituted Serum Matrix can be stored at ≤ -20°C for up to one month.

E. Preparation of Rat Cytokine Standard

1.) Prior to use, reconstitute the Rat Cytokine Standard with 250 µL deionized water to give a 20,000 pg/mL concentration of standard for all analytes except Leptin, which has a 100,000 pg/mL concentration after reconstitution. 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 original standard; the unused portion may be stored at ≤ -20°C for up to one month.

2.) Preparation of Working Standards

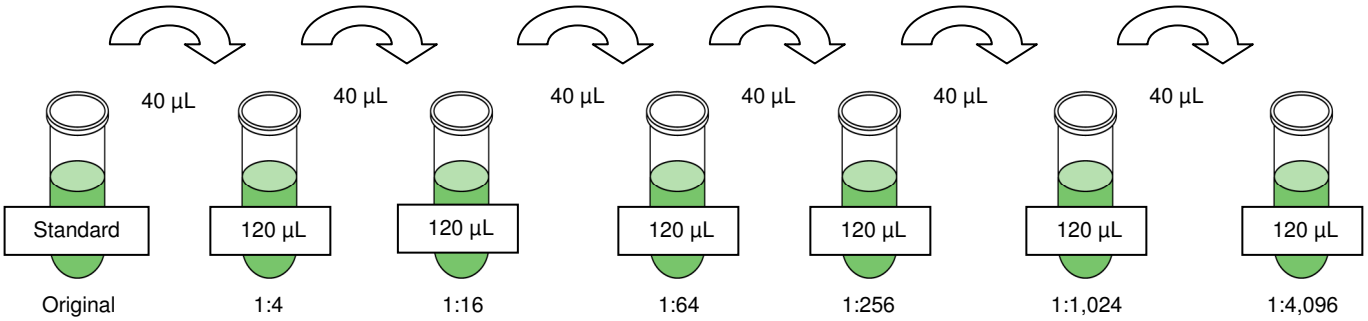
Label six polypropylene microfuge tubes 1:4, 1:16, 1:64, 1:256, 1:1,024 and 1:4,096. Add 120 µL of Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 40 µL of the original reconstituted standard to the 1:4 tube, mix well and transfer 40 µL of the 1:4 standard to the 1:16 tube, mix well and transfer 40 µL of the 1:16 standard to the 1:64 tube, mix well and transfer 40 µL of the 1:64 standard to 1:256 tube, mix well and transfer 40 µL of the 1:256 standard to the 1:1,024 tube and mix well, and transfer 40 µL of the 1:1,024 standard to the 1:4,096 tube and mix well. The 0 pg/mL standard (Background) will be Assay Buffer.

Based on individual needs, users may also use 3X or 5X serial dilutions for the standard curve.

Standard Tube (Dilution)	Volume of Deionized Water to Add	Volume of Standard to Add
Original	250 µL	0

Standard Tube (Dilution)	Volume of Assay Buffer to Add	Volume of Standard to Add
1:4	120 µL	40 µL of Original
1:16	120 µL	40 µL of 1:4
1:64	120 µL	40 µL of 1:16
1:256	120 µL	40 µL of 1:64
1:1,024	120 µL	40 µL of 1:256
1:4,096	120 µL	40 µL of 1:1,024

Standard Preparation:



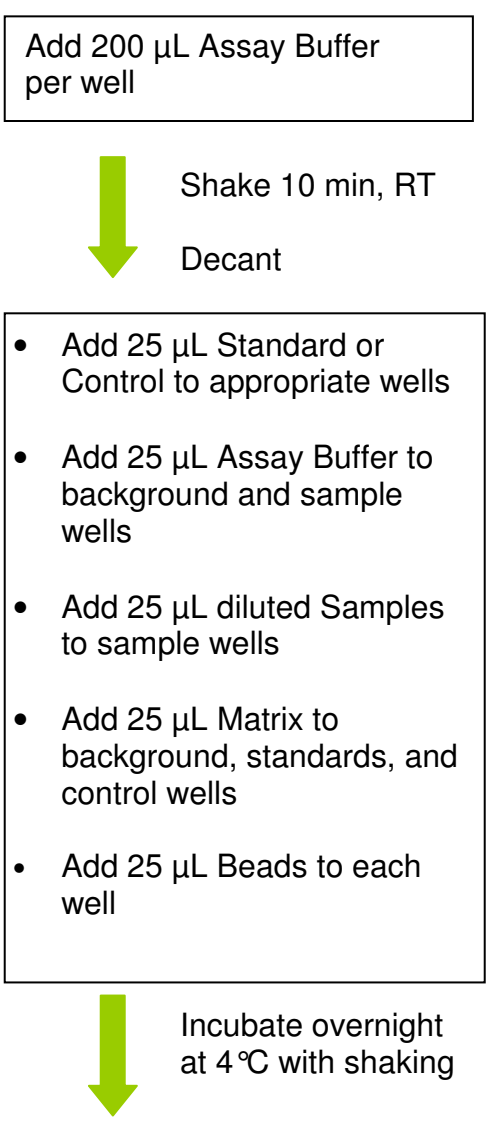
After serial dilutions, the tubes should have the following concentrations for constructing standard curves.

Standard Tube (Dilution)	Concentrations for All Analytes Except for Leptin	Concentration for Leptin
1:4,096	4.9 pg/mL	24.4 pg/mL
1:1,024	19.5 pg/mL	97.7 pg/mL
1:256	78.1 pg/mL	390.6 pg/mL
1:64	312.5 pg/mL	1,562.5 pg/mL
1:16	1,250 pg/mL	6,250 pg/mL
1:4	5,000 pg/mL	25,000 pg/mL
Original	20,000 pg/mL	100,000 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), 1:4,096, 1:1,024, 1:256, 1:64, 1:16, 1:4 and original], Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will only read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.
- If using a filter plate, set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Add 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for 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 5X diluted sample into the appropriate wells. (Alternatively, add 20 µL Serum Matrix to each sample well and 5 µL of undiluted sample to 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. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (18-20 hours) at 4°C.



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

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

Add 25 µL Detection Antibodies per well

Incubate 2 hours at RT

Do Not Aspirate

Add 25 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

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

Add 150 µL Sheath Fluid or Drive Fluid per well

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

PLATE WASHING

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

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

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

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

EQUIPMENT SETTINGS

Bio-Tek ELx405:

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

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

1.) Soak program:

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

2.) Wash program:

Method:

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

Dispense:

1. Dispense volume: 200 µL/well
2. Dispense flow rate: 5
3. Dispense height: 130 (16.51 mm)

4. Horizontal disp pos: 00 (0 mm)
5. Bottom Wash first?: NO
6. Prime before start?: NO

Aspiration:

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

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

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

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

Luminex 200™, HTS, FLEXMAP 3D™ and MAGPIX® with xPONENT software:

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

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

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

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

Events:	50, per bead	
Sample	100 µL	
Gate Settings:	8,000 to 15,000	
Reporter	Default (low PMT)	
Time	60 seconds	
Bead	Customizable 23-Plex	
	G-CSF	13
	Eotaxin	14
	GM-CSF	18
	IL-1α	19
	Leptin	22
	MIP-1α	33
	IL-4	34
	IL-1β	35
	IL-2	36
	IL-6	37
	IL-13	39
	IL-10	51
	IL-12p70	52
	IFNγ	53
	IL-5	55
	IL-17	56
	IL-18	57
	MCP-1	62
	IP-10	65
	GRO/KC	72
	VEGF	73
	TNFα	77
	RANTES	78

QUALITY CONTROLS

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located at the 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 for an analyte and any of the other analytes in this panel.

Assay Sensitivities (minimum detectable concentrations, pg/mL)

MinDC: Minimum Detectable Concentration is calculated using the Milliplex Analyst Software from Millipore. 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	MinDC* (pg/mL)
Eotaxin	13.6
G-CSF	3.0
GM-CSF	10.6
GRO/KC	12.2
IFNγ	13.7
IL-1α	15.0
IL-1β	4.9
IL-2	8.5
IL-4	12.5
IL-5	5.4
IL-6	28.7
IL-10	11.7

Cytokine	MinDC* (pg/mL)
IL-12p70	5.2
IL-13	3.56
IL-17	3.7
IL-18	6.5
IP-10	5.2
Leptin	18.2
MCP-1	15.1
MIP-1α	1.5
RANTES	14.6
TNFα	6.6
VEGF	14.8

*mean minDC + 2 standard deviations, N=6 assays

Precision

Intra-assay precision is generated from the mean of the %CV's from 8 reportable results across two different concentrations of cytokines in a single assay. Inter-assay precision is generated from the mean of the %CV's across two different concentrations of cytokines across 4 different assays.

Cytokine	Intra-Assay Precision (%CV)	Inter-Assay Precision (%CV)
Eotaxin	5.8	7.1
G-CSF	5.2	6.0
GM-CSF	7.5	9.2
GRO/KC	5.7	6.4
IFNγ	9.8	12.0
IL-1α	8.1	11.6
IL-1β	4.8	5.5
IL-2	8.5	8.4
IL-4	3.6	6.0
IL-5	5.9	8.3
IL-6	3.4	9.8
IL-10	8.7	12.2
IL-12p70	3.2	7.3
IL-13	6.7	9.2
IL-17	3.8	6.8
IL-18	5.4	6.3
IP-10	4.8	8.6
Leptin	15.8	13.9
MCP-1	4.3	9.7
MIP-1α	6.0	8.1
RANTES	6.5	10.6
TNFα	6.7	11.3
VEGF	4.2	7.0

Accuracy

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

Cytokine	Average % Recovery
Eotaxin	97
G-CSF	98
GM-CSF	93
GRO/KC	95
IFNγ	80
IL-1α	86
IL-1β	99
IL-2	95
IL-4	97
IL-5	90
IL-6	86
IL-10	85

Cytokine	Average % Recovery
IL-12p70	94
IL-13	102
IL-17	93
IL-18	98
IP-10	96
Leptin	101
MCP-1	86
MIP-1α	109
RANTES	91
TNFα	99
VEGF	93

TROUBLESHOOTING GUIDE

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

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

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

REPLACEMENT REAGENTS	**Catalog #**
Rat Cytokine Standard	LRC-8080
Rat Cytokine Quality Controls 1 & 2	LRC-6080
Bead Diluent	LA-BD
Serum Matrix	LMC-SD (optional)
Rat Cytokine Detection Antibodies	LRC-1080-1 LRC-1080-2 LRC-1080-3
Streptavidin-Phycoerythrin	L-SAPE4 (use with Cat.#LRC-1080-3) L-SAPE6 (use with Cat.#LRC-1080-1 or LRC-1080-2)
Assay Buffer	L-MAB
Set of two 96-Well plates with sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Magnetic Beads

Cytokine	*Bead #*	*Cat. #*
Eotaxin	*14*	*HETXN-MAG*
G-CSF	*13*	*MGCSF-MAG*
GM-CSF	*18*	*RGMCSF-MAG*
GRO/KC	*72*	*RGR0KC-MAG*
IFNγ	*53*	*RIFNG-MAG*
IL-1α	*19*	*RIL1A-MAG*
IL-1β	*35*	*RCYIL1B-MAG*
IL-2	*36*	*RIL2-MAG*
IL-4	*34*	*RIL4-MAG*
IL-5	*55*	*HIL5-MAG*
IL-6	*37*	*RCYIL6-MAG*
IL-10	*51*	*RIL10-MAG*
IL-12p70	*52*	*RIL12P70-MAG*
IL-13	*39*	*RIL13-MAG*
IL-17	*56*	*MIL17-MAG*
IL-18	*57*	*RIL18-MAG*
IP-10	*65*	*HIP10-MAG*
Leptin	*22*	*RCYLPTN-MAG*
MCP-1	*62*	*MCYMCP1-MAG*
MIP-1α	*33*	*RMIP1A-MAG*
RANTES	*78*	*RRNTS-MAG*
TNFα	*77*	*MCYTNFA-MAG*
VEGF	*73*	*RCYVEGF-MAG*
Premixed 23-plex Beads		*RCYPMX23-MAG*

ORDERING INFORMATION

To place an order:

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

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

FAX: (636) 441-8050
Toll Free US: (800) MILLIPORE
MAIL ORDERS: Millipore Corp.
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

For International Customers:

To best serve our international customers in placing an order or obtaining additional information about MILLIPLEX MAP products, please contact your multiplex specialist or sales representative or email our European Customer Service at customerserviceEU@Millipore.com.

Conditions of Sale

All products are for research use only. They are not intended for use in clinical diagnosis or for administration to humans or animals. All products are intended for *in vitro* use only.

Material Safety Data Sheets (MSDS)

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)	1:64 Standard	QC-1 Control	Etc.								
B	0 pg/mL Standard (Background)	1:64 Standard	QC-1 Control									
C	1:4,096 Standard	1:16 Standard	QC-2 Control									
D	1:4,096 Standard	1:16 Standard	QC-2 Control									
E	1:1,024 Standard	1:4 Standard	Sample 1									
F	1;1,024 Standard	1:4 Standard	Sample 1									
G	1:256 Standard	Original Standard	Sample 2									
H	1:256 Standard	Original Standard	Sample 2									