Rat Cytokine / Chemokine

96 Well Plate Assay

Cat. #: RCYTO-80K or RCYTO-80K-PMX, or RCYTO-80K-PMX23

MILLIPLEX® MAP

RAT CYTOKINE / CHEMOKINE KIT
96 Well Plate Assay

#RCYTO-80K or
#RCYTO-80K-PMX (14-plex premixed) or
#RCYTO-80K-PMX23 (premixed)

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FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

INTRODUCTION

"Cytokine" describes a diverse group of soluble proteins and peptides that 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 are not produced by specialized cells in glands. This includes lymphokines, interferons, colony stimulating factors and chemokines (cytokines with chemotactic activity).

Millipore is proud to announce that the former LINCO*plex* Rat Cytokine/Chemokine Panel now has the MILLIPLEX® MAP optimized format. While you will instantly recognize the quality and reproducibility that you have always trusted, you will also enjoy the enhancements that we have built into MILLIPLEX MAP.

Millipore's MILLIPLEX® MAP Rat Cytokine/Chemokine Panel is to be used for the simultaneous quantification of Eotaxin, G-CSF, GM-CSF, GRO/KC, IFNγ, IP-10, Leptin, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IL-18, MCP-1, MIP-1a, RANTES, TNFα and VEGF in rat plasma, serum, and cell/tissue culture supernatant samples. The panel provides quality tools for biomedical researchers who use rat animal models in study of inflammatory diseases.

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
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 premix panel)	LA-BD	4.0 mL	1 vial
Set of one 96-Well Filter Plate with 2 Sealers	MX-PLATE	----------	1 plate 2 sealers
Assay Buffer	L-MAB	30 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	1 bottle
Rat Cytokine Detection Antibodies	LRC-1080-1 or LRC-1080-2 or LRC-1080-3	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE4 *(Use with Cat. # LRC-1080-3)* or L-SAPE6 *(Use with Cat. # LRC-1080-1 & LRC-1080-2)*	3.2 mL	1 bottle
Mixing Bottle (not provided with premixed panel)	----------	----------	1 bottle

Rat Cytokine / Chemokine Antibody-Immobilized Premixed Beads:

Premixed 14-plex Beads	MXRCB-PMX	3.5 mL	1 bottle
Premixed 23-plex Beads	MXRCB-PMX23	3.5 mL	1 bottle

Included Rat Cytokine / Chemokine Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel (see following table page 4).

Rat Cytokine / Chemokine Antibody-Immobilized Beads:

Bead/Analyte Name	Luminex Bead Region	Customizable 23 Analytes (50X concentration, 90µL) Available	Cat. #	14-Plex Premixed Beads	23-Plex Premixed Beads
Anti-Rat Eotaxin Bead	2	✓	MXRETXN		✓
Anti-Rat GMCSF Bead	6	✓	MXRGM-CSF	✓	✓
Anti-Rat G-CSF Bead	7	✓	MXRG-CSF		✓
Anti-Rat IL-1α Bead	8	✓	MXRIL-1A	✓	✓
Anti-Rat MCP-1 Bead	13	✓	MXRMCP-1	✓	✓
Anti-Leptin Bead	16	✓	MXRME-LPTN		✓
Anti-Rat MIP-1α Bead	23	✓	MXRMIP-1A		✓
Anti-Rat IL-4 Bead	28	✓	MXRIL-4	✓	✓
Anti-Rat IL-1β Bead	34	✓	MXRIL-1B	✓	✓
Anti-Rat IL-2 Bead	36	✓	MXRIL-2	✓	✓
Anti-Rat IL-6 Bead	38	✓	MXRIL-6	✓	✓
Anti-Rat IL-13 Bead	45	✓	MXRIL-13		✓
Anti-Rat IL10 Bead	54	✓	MXRIL-10	✓	✓
Anti-Rat IL-12p70	56	✓	MXRIL-12	✓	✓
Anti-Rat IL-5 Bead	62	✓	MXRIL-5	✓	✓
Anti-Rat IFNγ Bead	64	✓	MXRIFN-G	✓	✓
Anti-Rat IL-17 Bead	67	✓	MXRIL-17		✓
Anti-Rat IL-18 Bead	69	✓	MXRIL-18	✓	✓
Anti-Rat IP-10 Bead	72	✓	MXRIP-10		✓
Anti-Rat GRO/KC Beads	73	✓	MXRGRO-KC	✓	✓
Anti-Rat RANTES Bead	75	✓	MXRRANTES		✓
Anti-Rat TNFα Bead	77	✓	MXRTNF-A	✓	✓
Anti-Rat VEGF Bead	99	✓	MXRVEGF		✓

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. Rubber Bands
6. Absorbent Pads
7. Laboratory Vortex Mixer
8. Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
9. Titer Plate Shaker (Lab-Line Instruments Model #4625 or equivalent)
10. Vacuum Filtration Unit (Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with Millipore Vacuum Pump Catalog #WP6111560 or equivalent)
11. Luminex 100™ IS, 200™, or HTS by Luminex Corporation
12. 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 ≤ -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. 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 ≤ -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 of 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 8 in Section IX. Immunoassay Procedure, following addition of 20 µL of the Serum Matrix to each sample well, 5 µL undiluted serum sample can be added directly to sample wells. **Measurement of RANTES and GRO/KC in some serum sample expected to have high concentrations may require further dilutions (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.
- EDTA is recommended as the anticoagulant in preparation of rat plasma samples.
- 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 of 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 8 in Section IX. Immunoassay Procedure, following addition of 20 µL of the Serum Matrix to each sample well, 5 µL undiluted plasma sample can be added directly to sample wells. **Measurement of RANTES and GRO/KC in some plasma samples expected to have high concentrations may require further dilutions (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.

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.

Example 1: When using 7 cytokine antibody-immobilized beads, add 60 µL from each of the 7 bead sets to the Mixing Bottle. Then add 2.58 mL Bead Diluent.

Example 2: When using 20 cytokine antibody-immobilized beads, add 60 µL from each of the 20 bead sets to the Mixing Bottle. Then add 1.8 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 should 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

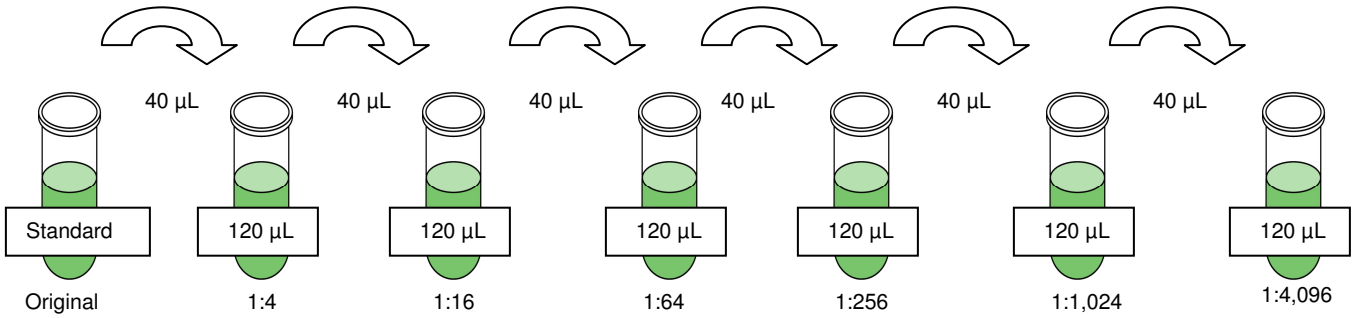
Label six polypropylene microfuge tubes 1:4, 1:16, 1:64, 1:256, 1:1,024 and 1:4,096. Add 120 µL of Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 40 µL of the original reconstituted standard to the 1:4 tube, mix well and transfer 40 µL of the 1:4 standard to the 1:16 tube, 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 Concentration (pg/mL)	Volume of Deionized Water to Add	Volume of Standard to Add
Original	250 µL	0

Standard Concentration (pg/mL)	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:



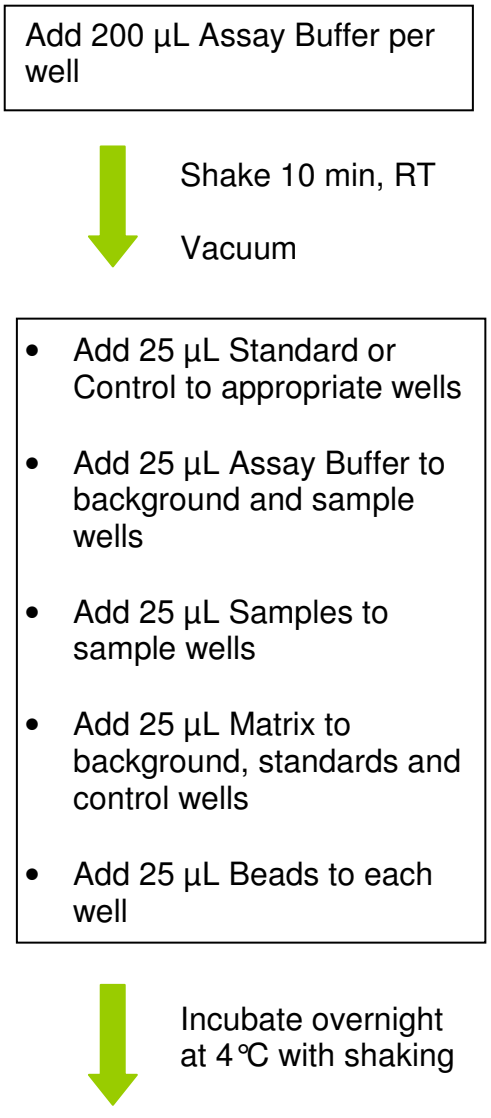
After serial dilutions, the tubes should have following concentrations for constructing standard curves.

Standard Tube (Dilution)	Concentrations for All Analytes Except for Leptin	Concentration for Leptin
Original	20,000 pg/mL	100,000 pg/mL
1:4	5,000 pg/mL	25,000 pg/mL
1:16	1,250 pg/mL	6,250 pg/mL
1:64	312.5 pg/mL	1,562.5 pg/mL
1:256	78.13 pg/mL	390.63 pg/mL
1:1,024	19.53 pg/mL	97.66 pg/mL
1:4,096	4.88 pg/mL	24.41 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), 1:4096, 1:1024, 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.
- 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 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 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for the 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 medium, use identical control medium as the matrix solution. When assaying tissue/cell culture extract, use identical extraction buffer as the matrix solution.
6. Add 25 µL of diluted sample 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 (18-20 hours) at 4°C.



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 2 hours 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™, or HTS.
19. Save and analyze the Median Fluorescent Intensity (MFI) data using a weighted 5 or 4-parameter logistic or spline curve-fitting method for calculating cytokine/chemokines concentrations in samples.

    When calculating final sample concentrations, remember to multiply by the dilution factor.

Vacuum and wash 2X with 200 µL Wash Buffer

Add 25 µL Detection Antibody per well

Incubate 2 hours 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	
Sample Size:	100 µL		100 µL		100 µL	
Gate Settings:	8,000 to 15,000					
Reporter Gain:	Default (Low PMT)					
Time Out:	60 seconds					
Bead Set:	14-Plex Premix Beads		23-Plex Premix Beads		Customizable 23-Plex Beads	
	rtGMCSF	6	rtEotaxin	2	rtEotaxin	2
	rtIL-1α	8	rtGMCSF	6	rtGMCSF	6
	rtMCP-1	13	rtGCSF	7	rtGCSF	7
	rtIL-4	28	rtIL-1α	8	rtIL-1α	8
	rtIL-1β	34	rtMCP-1	13	rtMCP-1	13
	rtIL-2	36	rtLeptin	16	rtLeptin	16
	rtIL-6	38	rtMIP-1α	23	rtMIP-1α	23
	rtIL-10	54	rtIL-4	28	rtIL-4	28
	rtIL-12p70	56	rtIL-1β	34	rtIL-1β	34
	rtIL-5	62	rtIL-2	36	rtIL-2	36
	rtIFNγ	64	rtIL-6	38	rtIL-6	38
	rtIL-18	69	rtIL-13	45	rtIL-13	45
	rtGRO/KC	73	rtIL-10	54	rtIL-10	54
	rtTNFα	77	rtIL-12p70	56	rtIL-12p70	56
			rtIL-5	62	rtIL-5	62
			rtIFNγ	64	rtIFNγ	64
			rtIL-17	67	rtIL-17	67
			rtIL-18	69	rtIL-18	69
			rtIP-10	72	rtIP-10	72
			rtGRO/KC	73	rtGRO/KC	73
			rtRANTES	75	rtRANTES	75
			rtTNFα	77	rtTNFα	77
			rtVEGF	99	rtVEGF	99

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

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	MinDC (pg/mL)
rtIL-1α	6.23
rtIL-1β	2.32
rtIL-2	3.67
rtIL-4	2.30
rtIL-5	2.89
rtIL-6	9.80
rtIL-10	5.41
rtIL-12p70	4.13
rtEotaxin	3.27
rtG-CSF	1.31
rtIP-10	3.78
rtLeptin	21.50

Cytokine	MinDC (pg/mL)
rtVEGF	4.93
rtIL-18	4.78
rtGMCSF	13.11
rtGRO/KC	2.06
rtIFNγ	4.88
rtMCP-1	3.81
rtTNFα	4.44
rtIL-13	23.2
rtIL-17	1.61
rtMIP-1α	1.94
rtRANTES	54.42

Precision

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

Cytokine	Intra-Assay Precision (%CV)	Interassay Precision (%CV)
rtIL-1α	5.80	10.2
rtIL-1β	7.53	12.4
rtIL-2	12.26	10.2
rtIL-4	5.93	8.8
rtIL-5	4.25	6.2
rtIL-6	10.37	14.3
rtIL-10	4.77	16.9
rtIL-12p70	6.53	11.2
rtIL-13	5.26	9.3
rtIL-17	5.96	5.9
rtIL-18	7.65	10.9
rtEotaxin	5.78	18.0
rtG-CSF	9.18	6.6
rtGMCSF	7.64	6.9
rtGRO/KC	7.39	14.1
rtIFNγ	5.91	14.7
rtIP-10	4.39	16.6
rtLeptin	8.15	8.5
rtMIP-1α	7.11	20.3
rtMCP-1	3.81	16.5
rtRANTES	8.34	17.7
rtTNFα	9.16	11.1
rtVEGF	9.16	8.3

Accuracy

Spike Recovery: The data represent mean percent recovery of 7 levels of spiked standards ranging from 31 to 2,000 pg/mL in serum matrices in 8 independent experiments.

Cytokine	Average % Recovery
rtIL-1α	89.97
rtIL-1β	77.97
rtIL-2	77.72
rtIL-4	88.92
rtIL-5	87.88
rtIL-6	89.63
rtIL-10	75.06
rtEotaxin	90.66
rtG-CSF	74.94
rtIP-10	91.35
rtLeptin	70.76

Cytokine	Average % Recovery
rtIL-12p70	94.71
rtIL-18	79.93
rtGMCSF	90.05
rtGRO/KC	78.63
rtIFNγ	87.59
rtMCP-1	76.30
rtTNFα	88.12
rtIL-13	83.26
rtIL-17	77.69
rtMIP-1 α	77.81
rtRANTES	71.67
rtVEGF	95.00

Cross-Reactivity

To test cross-reactivity among the assays in the panel, each cytokine was individually prepared at a concentration 5 times greater than the highest standard and tested as individual single-plex standards in the 14-plex assays using the multiplexed beads with immobilized capture antibodies and multiplexed detection antibody cocktail. There was none or negligible cross-reactivity among the antibodies with any of the other analytes in the panel.

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 pipetting 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 #**
Rat Cytokine/Chemokine Standard	LRC-8080
Rat Cytokine/Chemokine Quality Controls 1 & 2	LRC-6080
Serum Matrix	LMC-SD (optional)
Bead Diluent	LA-BD
Rat Cytokine Detection Antibodies	LRC-1080-1
	LRC-1080-2
	LRC-1080-3
Streptavidin-Phycoerythrin	L-SAPE4
	L-SAPE6
Assay Buffer	L-MAB
Set of two 96-Well Filter Plates with Sealers	MX-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Beads

Cytokine	Bead #	Cat. #
Eotaxin	2	MXRETXN
GMCSF	6	MXRGM-CSF
G-CSF	7	MXRG-CSF
RIL-1α	8	MXRIL-1A
MCP-1	13	MXRMCP-1
Leptin	16	MXRME-LPTN
MIP-1α	23	MXRMIP-1A
IL-4	28	MXRIL-4
IL-1β	34	MXRIL-1B
IL-2	36	MXRIL-2
IL-6	38	MXRIL-6
IL-13	45	MXRIL-13
IL-10	54	MXRIL-10

Cytokine	Bead #	Cat. #
IL-12p70	56	MXRIL-12
IL-5	62	MXRIL-5
IFNγ	64	MXRIFN-G
IL-17	67	MXRIL-17
IL-18	69	MXRIL-18
IP-10	72	MXRIP-10
GRO/KC	73	MXRGRO-KC
RANTES	75	MXRRANTES
TNF-α	77	MXRTNF-A
VEGF	99	MXRVEGF
Premixed 14-plex Beads		MXRCB-PMX
Premixed 23-plex Beads		MXRCB-PMX23

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WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/mL Standard (Background)	1:64 Standard	QC-I Control	Etc.								
B	0 pg/mL Standard (Background)	1:64 Standard	QC-I Control									
C	1:4,096 Standard	1:16 Standard	QC-II Control									
D	1:4,096 Standard	1:16 Standard	QC-II 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									