

**Non-Human Primate Cytokine
Magnetic Bead Panel II**

96-Well Plate Assay

**Cat. # PRCYT2MAG40K,
PRCYTMAG40K-PX24,
PRCYTMAG40K-PX25**

MILLIPLEX[®] MAP

NON-HUMAN PRIMATE CYTOKINE MAGNETIC BEAD PANEL II 96-Well Plate Assay

**# PRCYT2MAG40K,
PRCYTMAG40K-PX24 or
PRCYTMAG40K-PX25**

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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[®].

Non-Human Primate Cytokine Magnetic Bead Panel II

INTRODUCTION

Cytokines, immunomodulatory polypeptides that play key roles in both adaptive and innate immune responses, are one of the regulatory mechanisms of the immune system. Acting at the recognition, activation or effector phases of an immune response, cytokines modulate the development and functional activities of the subtypes of T cells, B cells and myeloid cells. Since chronic inflammation and aberrant immune response contribute to many disease states, research involving cytokines and chemokines plays a significant role in achieving a deeper understanding of the etiology and progression of diseases such as infectious disease, autoimmune and chronic inflammatory disease, cardiovascular disease, metabolic syndrome, neurological disorders and cancer.

Bead-based multiplex immunoassays enable precise, multiparametric analysis of the concurrent processes that underlie many diseases. Therefore, the MILLIPLEX[®] MAP Non-Human Primate Cytokine Panel II enables you to focus on the therapeutic potential of cytokines and chemokines, as well as the modulation of their expression. Coupled with the Luminex xMAP[®] platform, you receive the advantage of ideal speed and sensitivity, allowing quantitative multiplex detection of dozens of analytes simultaneously which can dramatically improve productivity.

EMD Millipore's MILLIPLEX[®] MAP Non-Human Primate Cytokine Magnetic Bead Panel II is the most versatile system available for immunology research.

- MILLIPLEX[®] MAP offers you the ability to:
 - Select a 24plex (for serum/plasma) or 25-plex (for cell culture) premixed kit or
 - Choose any combination of analytes from our panel of 25 analytes to design a custom kit that better meets your needs (Note: RANTES is for tissue culture samples only)
- A convenient "all-in-one" box format gives you the assurance that you will have all the necessary reagents you need to run your assay.

EMD Millipore's MILLIPLEX[®] MAP Non-Human Primate Cytokine Magnetic Bead Panel II is to be used for the simultaneous quantification of the following 25 non-human cytokines and chemokines : sCD137, Eotaxin, sFASL, FGF-2, Fractalkine, Granzyme A, Granzyme B, IL-1 α , IL-2, IL-4, IL-6, IL-16, IL-17A, IL-17E/IL-25, IL-21, IL-22, IL-23, IL-28A, IL-31, IL-33, IP-10, MIP-3 α , Perforin, RANTES and TNF β . (Note: RANTES is for tissue culture samples only and cannot be combined with other analytes in this panel when measuring serum or plasma due to different dilution needs.)

For Research Use Only. Not for Use in Diagnostic Procedures.

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, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres 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.
- EMD Millipore provides three Luminex instruments to acquire and analyze data using two detection methods:
 - The Luminex analyzers Luminex 200™ and FLEXMAP 3D[®], flow cytometry-based instruments that integrate key xMAP[®] detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
 - The Luminex analyzer (MAGPIX[®]), a CCD-based instrument that integrates key xMAP[®] capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. EMD Millipore combines the streamlined data acquisition power of Luminex xPONENT[®] acquisition software with sophisticated analysis capabilities of the new MILLIPLEX[®] Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex instruments.

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.
- For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze/thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin.**

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

Reagents Supplied	Catalog Number	Volume	Quantity
Non-Human Primate Cytokine Panel II Standard	PRCYT-8040	Lyophilized	1 vial
Non-Human Primate Cytokine Panel II Quality Controls 1 and 2	PRCYT-6040	Lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	MXHSM-8	Lyophilized	1 bottle
Set of one 96-Well Plate with 2 sealers	-----	-----	1 plate 2 sealers
Assay Buffer	L-AB	30 mL	2 bottles
Bead Diluent	LBD	3.5 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	2 bottles
Non-Human Primate Cytokine Panel II Detection Antibodies	PRCYT-1040	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE4	3.2 mL	1 bottle
Mixing Bottle (not provided with premixed panel)	-----	-----	1 bottle

Non-Human Primate Cytokine Panel II Antibody-Immobilized Premixed Magnetic Beads:

Premixed 24-plex Beads	PRCY2PMX24-MAG	3.5 mL	1 bottle
Premixed 25-plex Beads (Premixed 24 plex + RANTES)	PRCY2PMX24-MAG + HCYRNTS-MAG	3.5 mL	1 bottle + 1 vial

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

Non-Human Primate Cytokine Panel II Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable 25 Analytes (50X concentration, 90 µL) Available		Cat. #	24-Plex Magnetic Premixed Beads
Anti - FGF-2 Bead	13	✓		HCYFGF2-MAG	✓
Anti - Eotaxin Bead	14	✓		HETXN-MAG	✓
Anti - IL-16 Bead	18	✓		PRIL16-MAG	✓
Anti – Fractalkine Bead	21	✓		HFKN-MAG	✓
Anti - sCD137 Bead	22	✓		HSCD137-MAG	✓
Anti - MIP-3 α Bead	28	✓		HMIP3A-MAG	✓
Anti - Granzyme A Bead	29	✓		HGRNZMA-MAG	✓
Anti - IL-2 Bead	33	✓		PRIL2-MAG	✓
Anti - Granzyme B Bead	37	✓		HCDGRNZMB-MAG	✓
Anti - IL-17A Bead	39	✓		HIL17-MAG	✓
Anti - IL-22 Bead	43	✓		HIL22-MAG	✓
Anti - IL-1 α Bead	44	✓		HIL1A-MAG	✓
Anti - IL-33 Bead	45	✓		HIL33-MAG	✓
Anti - IL-21 Bead	52	✓		HIL21-MAG	✓
Anti- IL-4 Bead	53	✓		HIL4-MAG	✓
Anti - IL-23 Bead	54	✓		HIL23-MAG	✓
Anti -IL-6 Bead	57	✓		HCYIL6-MAG	✓
Anti - IP-10 Bead	65	✓		HIP10-MAG	✓
Anti - IL-17E/IL-25 Bead	66	✓		HIL17E-MAG	✓
Anti – sFasL Bead	67	✓		HCDSFASLG-MAG	✓
Anti - IL-31 Bead	73	✓		HIL31-MAG	✓
Anti – RANTES Bead	74	✓		HCYRNTS-MAG	
Anti – TNF β Bead	76	✓		HTNFB-MAG	✓
Anti - IL-28A Bead	77	✓		HIL28A-MAG	✓
Anti – Perforin Bead	78	✓		HPRFRN-MAG	✓

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

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

Instrumentation / Materials

1. Adjustable Pipettes with Tips capable of delivering 25 μ L to 1000 μ L
2. Multichannel Pipettes capable of delivering 5 μ L to 50 μ L or 25 μ L to 200 μ L
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. 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 (BioTek® 405 LS and 405 TS, EMD Millipore Catalog #40-094, #40-095, #40-096, #40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog #40-285 or equivalent).

Note: If a plate washer or handheld magnetic separation block for magnetic beads is not available, one can use a microtiter filter plate (EMD Millipore Catalog #MX-PLATE) to run the assay using a Vacuum Filtration Unit (EMD Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with EMD 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. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

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.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at $\leq -20^{\circ}\text{C}$ for 1 month and at $\leq -80^{\circ}\text{C}$ for greater than one month.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8°C for up to 1 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 analytes.
- 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 or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate or to the recommended EMD Millipore filter plates using 2 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. For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 μL Sheath Fluid in each well and 75 μL should be aspirated.
- 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.

TECHNICAL GUIDELINES (continued)

- For serum/plasma samples that require further dilution beyond 1:2, use the assay buffer provided in the kit.
- For cell/tissue homogenate, the final cell or tissue homogenate should be prepared in a buffer that has a neutral pH, contains minimal detergents or strong denaturing detergents, and has an ionic strength close to physiological concentration. Avoid debris, lipids, and cell/tissue aggregates. Centrifuge samples before use.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000xg. Remove serum and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$.
- Avoid multiple >2 freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Serum samples should be diluted 1:2 in the Assay Buffer provided in the kit. For example, in a tube, 35 μL of serum may be combined with 35 μL of Assay Buffer. When further dilution beyond 1:2 is required, use Assay Buffer as the diluent.
- **(Note: RANTES is for tissue culture samples only and cannot be combined with other analytes in this panel when measuring serum or plasma.)**

B. Preparation of Plasma Samples:

- Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1000xg within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$.
- Avoid multiple >2 freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Plasma samples should be diluted 1:2 in the assay buffer provided in the kit. For example, in a tube, 35 μL of serum may be combined with 35 μL of Assay Buffer. When further dilution beyond 1:2 is required, use assay buffer as the diluent.
- **(Note: RANTES is for tissue culture samples only and cannot be combined with other analytes in this panel when measuring serum or plasma.)**

C. Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$.
- Avoid multiple (>2) freeze/thaw cycles.
- Tissue culture supernatant may require a dilution with an appropriate control medium prior to assay. 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 **1:2 diluted** serum or plasma can be used. Tissue culture or other media may also be used.
- **Note: RANTES is for tissue culture samples only and cannot be combined with other analytes in this panel when measuring serum or plasma.**
- 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.

To prepare 25-plex premixed beads, add 70 μL of RANTES to the 24-plex premixed bead bottle. Mix well before use.

For individual vials of beads, sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 60 μL from each antibody-bead vial to the Mixing Bottle and bring final volume to 3.0 mL with LBD. Vortex the mixed beads well. Unused portion may be stored at 2-8 $^{\circ}\text{C}$ 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 3 antibody-immobilized beads, add 60 μL from each of the 3 bead vials to the Mixing Bottle. Then add 2.82 mL LBD.

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

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

B. Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 μ L deionized water. Invert the vial several times to mix and vortex. Allow the vial to sit for 5-10 minutes. Unused portion may be stored at $\leq -20^{\circ}\text{C}$ for up to one month.

C. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 60 mL of 10X Wash Buffer (two bottles) with 540 mL deionized water. Store the unused portion at $2-8^{\circ}\text{C}$ for up to one month.

D. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Add 1 mL deionized water to the bottle containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution. Leftover reconstituted Serum Matrix should be stored at $\leq -20^{\circ}\text{C}$ for up to one month.

E. Preparation of Non-Human Primate Cytokine Panel II Standard

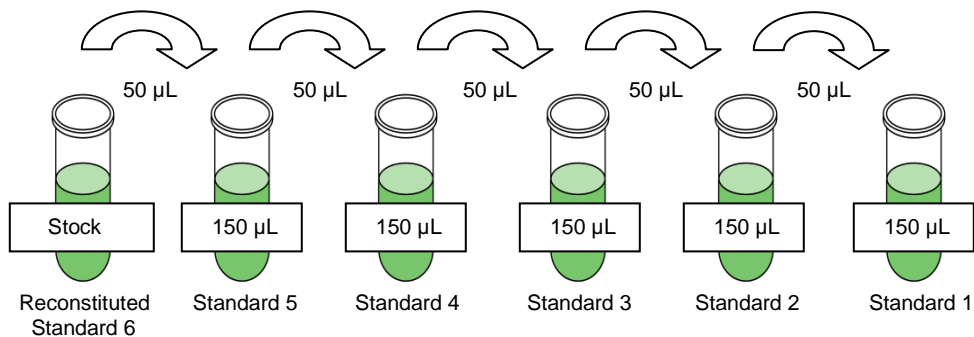
1.) Prior to use, reconstitute the Non-Human Primate Cytokine Panel II Standard with 250 μ L deionized water to give a stock concentration of standard for all analytes [see table below for analyte concentrations]. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. This will be used as the standard 6; the unused portion may be stored at $\leq -20^{\circ}\text{C}$ for up to one month.

2). Preparation of Working Standards

Label five polypropylene microfuge tubes as Standard 5, Standard 4, Standard 3, Standard 2 and Standard 1. Add 150 μ L of Assay Buffer to each of the five tubes. Prepare 1:4 serial dilutions by adding 50 μ L of the reconstituted Standard 6 to the Standard 5 tube, mix well and transfer 50 μ L of the Standard 5 to the Standard 4 tube, mix well and transfer 50 μ L of the Standard 4 to the Standard 3 tube, mix well and transfer 50 μ L of the Standard 3 to the Standard 2 tube, mix well and transfer 50 μ L of the Standard 2 to the Standard 1 tube. The 0 Standard (Background) will be the Assay Buffer.

Standard	Volume of Deionized Water to Add	Volume of Standard to Add
Standard 6	250 μ L	0

Standard Number	Volume of Assay Buffer to Add	Volume of Standard to Add
Standard 5	150 μ L	50 μ L of Standard 6
Standard 4	150 μ L	50 μ L of Standard 5
Standard 3	150 μ L	50 μ L of Standard 4
Standard 2	150 μ L	50 μ L of Standard 3
Standard 1	150 μ L	50 μ L of Standard 2



Standard	FGF2, IL-33 (pg/mL)	Eotaxin (pg/mL)	IL-16 (pg/mL)	Fractalkine, IL-22, TNF β , Perforin (pg/mL)	sCD137, IP-10, IL-2 (pg/mL)
Standard 1	20	3	73	98	10
Standard 2	78	12	293	391	39
Standard 3	313	47	1172	1563	156
Standard 4	1250	188	4688	6250	625
Standard 5	5000	750	18750	25000	2500
Standard 6	20000	3000	75000	100000	10000

Standard	MIP3 α (pg/mL)	Granzyme A (pg/mL)	IL-1 α IL-28A (pg/mL)	Granzyme B (pg/mL)	IL-17A (pg/mL)
Standard 1	7	78	29	4	1
Standard 2	27	313	117	16	6
Standard 3	109	1250	469	63	23
Standard 4	438	5000	1875	250	94
Standard 5	1750	20000	7500	1000	375
Standard 6	7000	80000	30000	4000	1500

Standard	IL-21, IL-31 (pg/mL)	IL-4 (pg/mL)	IL-23 (pg/mL)	IL-6 (pg/mL)	IL- 17E/IL-25 (pg/mL)
Standard 1	15	146	24	3	293
Standard 2	59	586	98	12	1172
Standard 3	234	2344	391	47	4688
Standard 4	938	9375	1563	188	18750
Standard 5	3750	37500	6250	750	75000
Standard 6	15000	150000	25000	3000	300000

Standard	sFASL (pg/mL)	RANTES (pg/mL)
Standard 1	8	1
Standard 2	31	3
Standard 3	125	12
Standard 4	500	47
Standard 5	2000	188

Standard 6	8000	750
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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), Standard 1 through 6], 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 as the matrix solution. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
6. Add 25 µL of 1:2 diluted Samples 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. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16–18 hours) at 4°C or same day 2 hrs at RT.

Add 200 µL Assay Buffer per well



Shake 10 min, RT

Decant

- Add 25 µL Standard or Control to appropriate wells
- Add 25 µL Assay Buffer to background wells and sample wells
- Add 25 µL appropriate matrix solution to background, standards, and control wells
- Add 25 µL 1:2 diluted Samples to sample wells
- Add 25 µL Beads to each well



Incubate overnight (16-18 hours) at 4°C or same day 2hrs at RT

9. Gently remove well contents and wash plate three 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 1 hour 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 three 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 analyte concentrations in samples. (Note: For diluted samples, multiply the calculated concentration by the dilution factor.)



Remove well contents and wash 3X with 200 μL Wash Buffer

Add 25 μL Detection Antibodies per well



Incubate 1 hour at RT (20-25°C) at RT

Do Not Aspirate

Add 25 μL Streptavidin-Phycoerythrin per well



Incubate for 30 minutes at RT

Remove well contents and wash 3X 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

If using a solid plate, use either a handheld magnet or magnetic plate washer.

1.) Solid Plate

If using a solid plate, use either a handheld magnet or magnetic plate washer.

- A.) Handheld magnet (**EMD Millipore Catalog #40-285**) - 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 μ L 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.) Magnetic plate washer (**EMD Millipore Catalog #40-094, #40-095, #40-096 and #40-097**) - Please refer to specific automatic plate washer manual for appropriate equipment settings. Please note that after the final aspiration, there will be approximately 25 μ L 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[®] 405 LS or 405 TS, please refer to the manufacturer's recommendations for programming instructions.

2.) Filter Plate (EMD Millipore Catalog #MX-PLATE)

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 μ L/well of Wash Buffer, removing Wash Buffer by vacuum filtration after each wash. Repeat wash steps as recommended in the Assay Procedure.

EQUIPMENT SETTINGS

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

These specifications are for the Luminex 200[™], Luminex HTS, Luminex FLEXMAP 3D[®], and Luminex MAGPIX[®] with xPONENT[®] software. Luminex instruments with other software (e.g. MasterPlex[®], STarStation, LiquiChip, Bio-Plex Manager[™], LABScan[™] 100) 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 (EMD Millipore Catalog #40-275) and performance verified with the Performance Verification Kit (EMD Millipore Catalog #40-276). The Luminex FLEXMAP 3D[®] instrument must be calibrated with the FLEXMAP 3D[®] Calibrator Kit (EMD Millipore Catalog #40-028) and performance verified with the FLEXMAP 3D[®] Performance Verification Kit (EMD Millipore Catalog #40-029). The Luminex MAGPIX[®] instrument must be calibrated with the MAGPIX[®] Calibration Kit (EMD Millipore Catalog #40-049) and performance verified with the MAGPIX[®] Performance Verification Kit (EMD Millipore Catalog #40-050).

NOTE: When setting up a Protocol using the xPONENT® software, you must select MagPlex as the Bead Type in the Acquisition settings.

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

EQUIPMENT SETTINGS (continued)

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

Events:	50, per bead	
Sample Size:	100 µL	
Gate Settings:	8,000 to 15,000	
Reporter Gain:	Default (low PMT)	
Time Out:	60 seconds	
Bead Set:	Customizable 25-Plex Beads	
	FGF-2	13
	Eotaxin	14
	IL-16	18
	Fractalkine	21
	sCD137	22
	MIP-3α	28
	Granzyme A	29
	IL-2	33
	Granzyme B	37
	IL-17A	39
	IL-22	43
	IL-1α	44
	IL-33	45
	IL-21	52
	IL-4	53
	IL-23	54
	IL-6	57
	IP-10	65
	IL-17E/IL-25	66
	sFasL	67
	IL-31	73
	RANTES	74
	TNFβ	76
	IL-28A	77
	Perforin	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 EMD Millipore Corporation 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)

Minimum Detectable Concentration (MinDC) is calculated using Milliplex Analyst. 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.

Analyte	Overnight Protocol (n = 9 Assays)		2 Hour Protocol (n = 4 Assays)	
	MinDC (pg/mL)	MinDC+2SD (pg/mL)	MinDC (pg/mL)	MinDC+2SD (pg/mL)
FGF-2	12.0	18.4	11.3	18.0
Eotaxin	2.5	4.5	2.5	3.1
IL-16	36.9	84.4	64.3	73.2
Fractalkine	32.6	69.2	27.1	65.1
sCD137	5.3	10.0	3.2	10.4
MIP-3 α	4.3	8.5	3.6	8.5
Granzyme A	13.6	24.3	10.2	18.8
IL-2	4.4	9.2	5.5	10.6
Granzyme B	1.9	3.4	1.6	2.9
IL-17A	1.8	3.1	1.7	3.6
IL-22	92.0	100.1	76.4	142.1
IL-1 α	23.7	40.2	14.3	39.7
IL-33	6.2	14.2	4.0	12.5
IL-21	5.9	11.9	6.3	14.9
IL-4	38.5	136.1	61.9	153.6
IL-23	7.5	11.4	7.1	17.9
IL-6	7.1	17.8	1.4	3.5
IP-10	5.6	10.3	5.9	9.8
IL-17E/IL-25	252.9	366.0	266.6	296.6
sFasL	5.5	11.3	5.9	12.6
IL-31	7.2	14.5	6.8	16.6
RANTES	0.7	2.0	1.7	2.2
TNF β	43.9	106.2	39.0	92.5
IL-28A	14.0	30.8	12.8	33.8
Perforin	35.8	98.3	38.9	61.2

Precision

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

Analyte	Overnight Protocol		2 Hour Protocol
	Intra-assay %CV	Inter-assay %CV	Intra-assay %CV
FGF-2	<10	<15	<10
Eotaxin	<10	<15	<10
IL-16	<10	<15	<10
Fractalkine	<10	<15	<10
sCD137	<10	<15	<10
MIP-3 α	<10	<15	<10
Granzyme A	<10	<15	<10
IL-2	<10	<15	<10
Granzyme B	<10	<15	<10
IL-17A	<10	<15	<10
IL-22	<10	<15	<10
IL-1 α	<10	<15	<10
IL-33	<10	<15	<10
IL-21	<10	<15	<10
IL-4	<10	<15	<10
IL-23	<10	<15	<10
IL-6	<10	<15	<10
IP-10	<10	<15	<10
IL-17E/IL-25	<10	<15	<10
sFasL	<10	<15	<10
IL-31	<10	<15	<10
RANTES	<10	<15	<10
TNF β	<10	<15	<10
IL-28A	<10	<15	<10
Perforin	<10	<15	<10

ASSAY CHARACTERISTICS (continued)

Accuracy

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

Analyte	Overnight Protocol	2 Hour Protocol
	% Recovery in Serum Matrix	% Recovery in Serum Matrix
FGF-2	99	77
Eotaxin	94	87
IL-16	89	83
Fractalkine	101	87
sCD137	100	87
MIP-3 α	104	88
Granzyme A	97	86
IL-2	93	78
Granzyme B	102	85
IL-17A	104	81
IL-22	96	78
IL-1 α	98	82
IL-33	106	87
IL-21	92	90
IL-4	104	80
IL-23	95	95
IL-6	88	74
IP-10	99	86
IL-17E/IL-25	109	92
sFasL	86	77
IL-31	101	86
RANTES	80	80
TNF β	98	87
IL-28A	97	91
Perforin	99	86

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Insufficient bead count	<p>Plate washer aspirate height set too low</p> <p>Bead mix prepared inappropriately</p> <p>Samples cause interference due to particulate matter or viscosity</p> <p>Probe height not adjusted correctly</p>	<p>Adjust aspiration height according to manufacturers' instructions.</p> <p>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.</p> <p>See above. Also sample probe may need to be cleaned with alcohol flushes, back flushes and washes; or, if needed, probe should be removed and sonicated.</p> <p>When reading the assay on Luminex 200™, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height to the kit solid plate using 1 alignment disc. For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.</p>
Background is too high	<p>Background wells were contaminated</p> <p>Matrix used has endogenous analyte or interference</p> <p>Insufficient washes</p>	<p>Avoid cross-well contamination by using sealer appropriately and pipetting with multichannel pipettes without touching reagent in plate.</p> <p>Check matrix ingredients for cross-reacting components (e.g. interleukin modified tissue culture medium).</p> <p>Increase number of washes.</p>
Beads not in region or gate	<p>Luminex instrument not calibrated correctly or recently</p> <p>Gate settings not adjusted correctly</p> <p>Wrong bead regions in protocol template</p> <p>Incorrect sample type used</p> <p>Instrument not washed or primed</p> <p>Beads were exposed to light</p>	<p>Calibrate Luminex instrument based on manufacturer's instructions, at least once a week or if temperature has changed by >3°C.</p> <p>Some Luminex instruments (e.g. Bio-Plex®) require different gate settings than those described in the kit protocol. Use instrument default settings.</p> <p>Check kit protocol for correct bead regions or analyte selection.</p> <p>Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required.</p> <p>Prime the Luminex instrument 4 times to rid it of air bubbles, wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid.</p> <p>Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.</p>

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

	High lipid concentration	After centrifugation, remove lipid layer and use supernatant.
Plate leaked	Vacuum pressure too high	Adjust vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (blocked) plate and continue.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate holder or raised edge so bottom of filter is not touching any surface.
	Insufficient blotting of filter plate bottom 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

Non-Human Primate Cytokine Panel II Standard	PRCYT-8040
Non-Human Primate Cytokine Panel II Quality Controls 1 and 2	PRCYT-6040
Serum Matrix	MXHSM-8
Non-Human Primate Cytokine Panel II Detection Antibodies	PRCYT-1040
Streptavidin-Phycoerythrin	L-SAPE4
Assay Buffer	L-AB
Set of two 96-Well plates with sealers	MAG-PLATE
10X Wash Buffer	L-WB
Bead Diluent	LBD

Non-Human Primate Cytokine Panel II Antibody-Immobilized Premixed Magnetic Beads:

Premixed 24-plex Beads	PRCY2PMX24-MAG	3.5 mL
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Antibody-Immobilized Magnetic Beads

<u>Analyte</u>	<u>Bead #</u>	<u>Cat. #</u>
FGF-2	13	HCFYFGF2-MAG
Eotaxin	14	HETXN-MAG
IL-16	18	PRIL16-MAG
Fractalkine	21	HFKN-MAG
sCD137	22	HSCD137-MAG
MIP-3 α	28	HMIP3A-MAG
Granzyme A	29	HGRNZMA-MAG
IL-2	33	PRIL2-MAG
Granzyme B	37	HCDGRNZMB-MAG
IL-17A	39	HIL17-MAG
IL-22	43	HIL22-MAG
IL-1 α	44	HIL1A-MAG
IL-33	45	HIL33-MAG
IL-21	52	HIL21-MAG
IL-4	53	HIL4-MAG
IL-23	54	HIL23-MAG
IL-6	57	HCYIL6-MAG
IP-10	65	HIP10-MAG
IL-17E/IL-25	66	HIL17E-MAG
sFasL	67	HCDSFASLG-MAG
IL-31	73	HIL31-MAG
RANTES	74	HCYRNTS-MAG
TNF β	76	HTNFB-MAG
IL-28A	77	HIL28A-MAG
Perforin	78	HPRFRN-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:

Include:

- 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: (866) 441-8400
(636) 441-8400

Mail Orders: EMD Millipore Corporation
6 Research Park Drive
St. Charles, Missouri 63304 U.S.A.

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Conditions of Sale

For Research Use Only. Not for Use in Diagnostic Procedures.

Material Safety Data Sheets (MSDS)

Material Safety Data Sheets for EMD Millipore products may be ordered by fax or phone or through our website at www.emdmillipore.com/techlibrary/index.do.

Technical Services

<http://www.emdmillipore.com/techservices>

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For North America: Toll-Free US: 1-(800) 221-1975 or 1-(781) 533-8045

Outside North America, contact your local office

<http://www.emdmillipore.com/offices>

WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard 0 (Background)	Standard 4	QC-2 Control									
B	Standard 0 (Background)	Standard 4	QC-2 Control									
C	Standard 1	Standard 5	Sample 1									
D	Standard 1	Standard 5	Sample 1									
E	Standard 2	Standard 6	Sample 2									
F	Standard 2	Standard 6	Sample 2									
G	Standard 3	QC-1 Control	Etc.									
H	Standard 3	QC-1 Control										