

Fluorokine[®] MAP

Mouse MultiAnalyte Profiling Base Kit

Catalog Number LUM000

For the simultaneous quantitative determination of multiple mouse cytokine concentrations in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.

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

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INTRODUCTION

Cytokines are intercellular signaling proteins released from a wide variety of cells and tissues. They play an integral role in regulating growth and cellular proliferation as well as modulating host response to infection, injury, and inflammation. Cytokines also influence reproduction and bone remodeling. A large number of cytokines are pleiotropic and share similar functions. In addition, many cytokines influence the production of other cytokines. Analysis and quantitation of cytokines within biological fluids and cell culture supernates has thus become increasingly important. Methods such as bioassay, enzyme-linked immunosorbent assay (ELISA), intracellular staining, ribonuclease protection assay (RPA) and polymerase chain reaction (PCR) have all been used for quantifying cytokines; however, each of these techniques has limitations associated with it. These techniques are not capable of measuring multiple cytokines simultaneously in a limited sample volume.

Any combination of the following bead sets is suitable for use with the Fluorokine MAP Mouse Base Kit.

Analyte	Catalog Number	Microparticle Region
CCL2/JE	LUM479	30
GM-CSF	LUM415B	11
IFN- γ	LUM485	75
IL-1 β /IL-1F2	LUM401	06
IL-2	LUM402	17
IL-4	LUM404	21
IL-5	LUM405	54
IL-6	LUM406	32
IL-10	LUM417	50
IL-12 p70	LUM419	66
IL-13	LUM413	36
IL-17	LUM421	39
KC	LUM453	26
MIP-2	LUM452	47
TNF- α /TNFSF1A	LUM410	79
VEGF	LUM493	43

PRINCIPLE OF THE ASSAY

Fluorokine MAP multiplex kits are designed for use with a Luminex[®] 100™, Luminex 200™, or Bio-Rad[®] Bio-Plex[®], dual laser, flow-based sorting and detection analyzer manufactured by Luminex Corporation.

Analyte-specific antibodies are pre-coated onto color-coded microparticles. Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated detection antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds the biotinylated detection antibodies, is added to each well. A final wash removes unbound Streptavidin-PE and the microparticles are resuspended in buffer and read using a Luminex analyzer. One laser is microparticle-specific and determines which analyte is being detected. The other laser determines the magnitude of the phycoerythrin-derived signal, which is in direct proportion to the amount of analyte bound.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins and other factors present in biological samples. However, until these proteins have been tested in Fluorokine MAP, the possibility of interference cannot be excluded.
- Fluorokine MAP affords the user the benefit of multianalyte analysis in a complex sample. For each sample type, a single, multipurpose diluent is used to optimize recovery, linearity and reproducibility. Such a multipurpose diluent may not optimize any single analyte to the same degree that a unique diluent selected for analysis of that analyte can optimize conditions. Therefore, some performance characteristics may be more variable than those for assays designed specifically for single analyte analysis.
- **Only analytes listed on the enclosed Standard Value Card can be measured with this base kit.**

MATERIALS PROVIDED

Standard Cocktail 1 (Part 895814) - 2 vials of recombinant mouse cytokines in a buffered protein base with preservatives; lyophilized.

Standard Value Card (Part 750601) - 1 card listing the Standard Cocktail reconstitution volume and concentrations for this lot of base kit.

Microparticle Diluent 2 (Part 895815) - 6 mL of a buffered protein base with preservatives.

Calibrator Diluent RD5K Concentrate (Part 895119) - 21 mL of a 2-fold concentrated solution of a buffered protein base with preservatives. *For cell culture supernate samples.*

Calibrator Diluent RD6-40 (Part 895817) - 21 mL of a buffered protein base with preservatives. *For serum/plasma samples.*

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.

Biotin Antibody Diluent (Part 895816) - 5.25 mL of a buffered protein base with preservatives.

Streptavidin-PE (Part 892525) - 0.07 mL of a 100-fold concentrated streptavidin-phycoerythrin conjugate with preservatives.

Microplate (Part 640763) - 1 filter-bottomed plate, used as a vessel for the assay.

Plate Sealers (Part 640445) - 4 adhesive strips.

Mixing Bottles (Part 895505) - 2 (8 mL) empty bottles used for mixing microparticles with Microparticle Diluent.

PRECAUTION

The Biotin Antibody Diluent in this kit contains sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Biotin Antibody Diluent	
	Calibrator Diluent RD5K (1X)	
	Calibrator Diluent RD6-40	
	Microparticle Diluent 2	
	Diluted Streptavidin-PE	
	Diluted Biotin Antibody Cocktail	
	Undiluted Microparticles	
	Diluted Microparticle Mixture	
	Standard Cocktail 1	Discard after use. Use a fresh standard for each assay.

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- **Fluorokine MAP analyte-specific kit(s) (see page 2).**
- Luminex 100, Luminex 200, or Bio-Rad Bio-Plex analyzer with X - Y platform.
- Microplate vacuum manifold (Millipore Multiscreen™ Vacuum Manifold, Catalog # MAVM096, or equivalent).
- Pipettes and pipette tips.
- Deionized or distilled water.
- Multi-channel pipette, manifold dispenser, or automated dispensing unit.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Microcentrifuge.
- **Polypropylene** test tubes for dilution.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature or overnight at $2 - 8^{\circ}$ C before centrifuging for 15 minutes at $1000 \times g$. Remove serum and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA as an anticoagulant. Centrifuge for 15 minutes at $1000 \times g$ within 30 minutes of collection. Assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Serum and plasma samples require a 4-fold dilution. A suggested 4-fold dilution is $30 \mu\text{L}$ of sample + $90 \mu\text{L}$ of Calibrator Diluent RD6-40. Mix thoroughly.

REAGENT PREPARATION

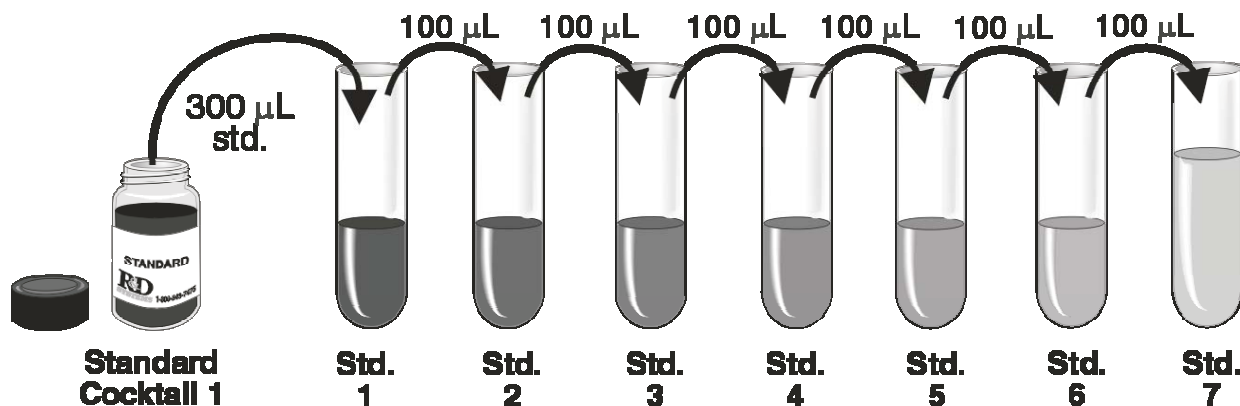
Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent RD5K (1X) - Dilute 20 mL of Calibrator Diluent RD5K Concentrate into deionized or distilled water to prepare 40 mL of Calibrator Diluent RD5K (1X).

Standard - Reconstitute the Standard Cocktail 1 with Calibrator Diluent RD5K (1X) (*for cell culture supernate samples*) or Calibrator Diluent RD6-40 (*for serum/plasma samples*). Refer to the Standard Value Card for the reconstitution volume. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 300 μL of reconstituted Standard Cocktail 1 into the working standard 1 tube. Pipette 200 μL of the appropriate Calibrator Diluent into the remaining tubes. Use working standard 1 to produce a 3-fold dilution series (below). Mix each tube thoroughly before the next transfer. Working standard 1 serves as the high standard. The appropriate Calibrator Diluent serves as the blank. Refer to the Standard Value card for the assigned values of working standard 1.



DILUTED MICROPARTICLE MIXTURE PREPARATION

1. Centrifuge each Microparticle Concentrate vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vials to resuspend the microparticles, taking precautions not to invert the vials.
3. Dilute the Microparticle Concentrates in the mixing bottle provided. The volume of the Microparticle Concentrate listed in the table below is for each analyte (*e.g.* if measuring a full plate of IL-1 β and IL-6, add 50 μL of IL-1 β Microparticle Concentrate and 50 μL of IL-6 Microparticle Concentrate to 5 mL of Microparticle Diluent).

# Wells used	Microparticle Concentrate(s)	+	Microparticle Diluent
96	50 μL	+	5.0 mL
72	37.5 μL	+	3.75 mL
48	25 μL	+	2.5 mL
24	12.5 μL	+	1.25 mL

Note: Protect microparticles from light during handling. Diluted microparticles cannot be stored. Prepare microparticles within 30 minutes of use.

DILUTED BIOTIN ANTIBODY COCKTAIL PREPARATION

1. Centrifuge each Biotin Antibody vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vials, taking precautions not to invert the vials.
3. Add 50 μ L of each Biotin Antibody concentrate to the vial of Biotin Antibody Diluent. Mix gently.

STREPTAVIDIN-PE PREPARATION

Use a polypropylene amber bottle or a polypropylene tube covered with aluminum foil. Protect Streptavidin-PE from light during handling and storage.

1. Centrifuge the Streptavidin-PE vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Dilute the 100X Streptavidin-PE to a 1X concentration by adding 55 μ L of Streptavidin-PE to 5.5 mL of Wash Buffer.

INSTRUMENT SETTINGS

Adjust the probe height setting on the Luminex analyzer to avoid puncturing the membrane. Refer to the instrument manual.

- a) Assign a bead region for each analyte being measured
- b) 50 events/bead
- c) Minimum events: 0
- d) Flow rate: 60 μ L/Min (fast)
- e) Sample size: 50 μ L
- f) Doublet Discriminator gates at approximately 7500 and 15500
- g) Collect Median RFU

Note: For the Bio-Rad Bio-Plex Analyzer, set the gates at 4300 and 10000. The CAL2 setting for the Bio-Rad Bio-Plex Analyzer should be set at the low RP1 target value.

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples and standards be assayed in duplicate.

Note: *Protect microparticles and Streptavidin-PE from light at all times.*

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Pre-wet the filter-bottomed microplate by filling each well with 100 μL of Wash Buffer. Remove the liquid through the filter at the bottom of the plate using a vacuum manifold designed to accommodate a microplate.
3. Resuspend the diluted microparticle mixture by inversion or vortexing. Add 50 μL of the microparticle mixture to each well of the filter-bottomed microplate provided.
4. Add 50 μL of Standard or sample* per well. Pipet assay within 20 minutes. Securely cover with a foil plate sealer. Incubate for 3 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.
5. Using a vacuum manifold device designed to accommodate a microplate, wash by removing the liquid, filling each well with Wash Buffer (100 μL) and removing the liquid again. All of the liquid must be removed through the filter at the bottom of the plate to avoid any loss of microparticles. Complete removal of liquid is essential for good performance. Perform the wash procedure three times.
6. Add 50 μL of diluted Biotin Antibody Cocktail to each well. Securely cover with a foil plate sealer and incubate for 1 hour at room temperature on the shaker set at 500 ± 50 rpm.
7. Repeat the wash as in step 5.
8. Add 50 μL of diluted Streptavidin-PE to each well. Securely cover with a foil plate sealer and incubate for 30 minutes at room temperature on the shaker set at 500 ± 50 rpm.
9. Repeat the wash as in step 5.
10. Resuspend the microparticles by adding 100 μL of Wash Buffer to each well. Incubate for 2 minutes on the shaker set at 500 ± 50 rpm.
11. Read within 90 minutes using a Luminex or Bio-Rad analyzer.

*Serum/plasma samples require dilution. See Sample Preparation section.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and standards as instructed.



2. Pre-wet the plate by filling each well with 100 μ L of Wash Buffer. Remove the liquid through the filter at the bottom of the plate using a vacuum manifold designed to accommodate a microplate.



3. Add 50 μ L of diluted microparticle mixture to each well.



4. Add 50 μ L Standard or sample* to each well. Incubate 3 hours at RT on a horizontal orbital microplate shaker.



5. Wash by removing the liquid from each well, filling each well with Wash Buffer (100 μ L), and removing the liquid again. Perform the wash 3 times.



6. Add 50 μ L diluted Biotin Antibody Cocktail to each well. Incubate 1 hour at RT on the shaker.



7. Repeat the wash as in step 5.



8. Add 50 μ L diluted Streptavidin-PE to each well. Incubate 30 minutes at RT on the shaker.



9. Repeat the wash as in step 5.



10. Add 100 μ L Wash Buffer to each well. Incubate 2 minutes at RT on the shaker.



11. Read within 90 minutes using a Luminex or Bio-Rad analyzer. Refer to the Instrument Settings section.

**Protect microparticles and Streptavidin-PE
from light at all times.**

*Serum/plasma samples require dilution.

CALCULATION OF RESULTS

Use the Standard concentrations on the Standard Value Card as the high standard and calculate 3-fold dilutions for the remaining levels. Average the duplicate readings for each standard and sample and subtract the average blank median RFU.

Create a standard curve for each analyte by reducing the data using computer software capable of generating a 5-PL curve fit. As an alternative, construct a standard curve by plotting the median RFU for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the analyte concentrations versus the log of the RFU and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

To determine the concentration of each analyte, first find the RFU value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding concentration.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Protect microparticles and Streptavidin-PE from light at all times to prevent photobleaching.
- For best results, adjust the vacuum strength to between 15 and 40 cm of mercury.

CALIBRATION

This assay is calibrated against highly purified recombinant mouse cytokines produced at R&D Systems.

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Luminex is a registered trademark of Luminex Corporation.
Luminex 100 and Luminex 200 are trademarks of Luminex Corporation.
Multiscreen is a trademark of Millipore Corporation.*

PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.

1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
	A	B	C	D	E	F	G	H

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