Mouse Cytokine/Chemokine Magnetic Bead Panel II

96 Well Plate Assay

Cat. # MCYP2MAG-73K
Cat. # MCP2MG-73K-PX12

MILLIPLEX® MAG

Mouse CYTOKINE / CHEMOKINE Magnetic Bead Panel II KIT
96 Well Plate Assay

MCYP2MAG-73K-xx
or # MCP2MG-73K-PX12 (12-plex premixed)

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

INTRODUCTION

"Cytokine" is a general term used for a diverse group of soluble proteins and peptides which act as regulators under both normal and pathological conditions to modulate the functional activities of individual cells and tissues. These proteins also mediate direct interactions between cells and regulate processes taking place in the extracellular environment. Cytokines differ from hormones in that they act on a wider spectrum of target cells. Also, unlike hormones, they are not produced by specialized cells which are organized in specialized glands. The cytokine group of proteins includes lymphokines, interferons, colony stimulating factors and chemokines. Cytokine and chemokine research plays a significant role in achieving a deeper understanding of the immune system and its multi-faceted response to most antigens, as well as disease states such as inflammatory disease, allergic reactions, IBD, sepsis, and cancer.

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

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

Therefore, the **MILLIPLEX® *MAG*** Mouse Cytokine / Chemokine Panel II enables you to focus on the therapeutic potential of cytokines as well as the modulation of cytokine expression. Coupled with the Luminex xMAP® platform in a **magnetic bead** format, you receive the advantage of ideal speed and sensitivity, allowing quantitative multiplex detection of multiple analytes simultaneously, which can dramatically improve productivity.

Millipore's MILLIPLEX™ Mouse Cytokine / Chemokine Panel II is an extension panel of Mouse Cytokine/Chemokine panel, which is the most versatile system available for cytokine and chemokine research.

- MILLIPLEX™ MAP offers you the ability to:
  - Select a premixed kit (12plex).
  - Choose any combination of analytes from our panel of 12 analytes to design a custom kit that better meets your needs.
- A convenient "all-in-one" box format gives you the assurance that you will have all the necessary reagents you need to run your assay.

Millipore's MILLIPLEX™ Mouse Cytokine / Chemokine Panel II kit is to be used for the simultaneous quantification of the following 12 Mouse cytokines and chemokines: Erythropoietin(EPO), Exodus-2(CCL21/6Ckine), MCP-5(CCL12), MIP-3β(CCL19), TARC(CCL17), MIP-3a(CCL20), IL-16, Fractalkine(CX3CL1), IL-21, IL-22, IL-25(IL-17E), and IL-28B.

INTRODUCTION (continued)

This kit may be used for the analysis of all or any combination of the above cytokines and chemokines in serum, plasma, tissue/cell lysate and culture supernatant samples.

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 & MILLIPLEX *MAG* are based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life-sciences, and is 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 two 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 RECONSITUTED STANDARDS OR CONTROLS IN GLASS VIALS**. For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
Mouse Cytokine / Chemokine Panel II Standard	MXM8073	Lyophilized	1 vial
Mouse Cytokine Panel II Quality Controls 1 and 2	MXM6073	Lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	MXMSM	Lyophilized	1 vial
Set of two 96-Well Plates with 4 Sealers	MAG-PLATE		2 plates 4 sealers
Assay Buffer	L-MAB	30 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	2 bottles
Mouse Cytokine Panel II Detection Antibodies	MXM1073	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE3	3.2 mL	1 bottle
Mixing Bottle (not provided with premixed panel)	----------	----------	1 bottle

Mouse Cytokine / Chemokine Panel II Antibody-Immobilized Premixed Magnetic Beads:

Premixed 12-plex Beads	MP2PMX12-MAG	3.5 mL	1 bottle

Mouse Cytokine / Chemokine Panel II Antibody-Immobilized Beads:

Bead/Analyte Name	Luminex Bead Region	Customizable 12 Analytes (50X concentration, 90µL) Available	Cat. #	12-Plex Premixed Beads
Anti-Mouse EPO Bead	12	✓	MEP0-MAG	✓
Anti-Mouse Exodus-2 Bead	14	✓	MEXDS2-MAG	✓
Anti-Mouse MCP-5 Bead	18	✓	MMCP5-MAG	✓
Anti-Mouse MIP-3β Bead	20	✓	MMIP3B-MAG	✓
Anti-Mouse MIP-3a Bead	22	✓	MMIP3A-MAG	✓
Anti-Mouse TARC Bead	27	✓	MTARC-MAG	✓
Anti-Mouse IL-16 Bead	29	✓	MIL16-MAG	✓
Anti-Mouse Fractakine Bead	33	✓	MFKN-MAG	✓
Anti-Mouse IL-21 Bead	35	✓	MIL21-MAG	✓
Anti-Mouse IL-22 Bead	37	✓	MIL22-MAG	✓
Anti-Mouse IL-25 Bead	39	✓	MIL25-MAG	✓
Anti-Mouse IL-28B Bead	42	✓	MIL28B-MAG	✓

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (Luminex Catalogue #40-50000)

Instrumentation / Materials

1. Adjustable Pipettes with Tips capable of delivering 25 µL to 1000 µl
2. Multichannel Pipettes capable of delivering 5 µl to 50 µl or 25 µl to 200 µl
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. Aluminum Foil
6. Rubber Bands
7. Laboratory Vortex Mixer
8. Sonicator (Branson Ultrasonic Cleaner Model # B200 or equivalent)
9. Titer Plate Shaker (Lab-Line Instruments, Model #4625, or equivalent)
10. Luminex $^{200,\ HTS}$ or FLEXMAP 3D™ with xPONENT software by Luminex Corporation
11. Plate washer for magnetic beads (Bio-Tek ELx405, 40-015)

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

SAFETY PRECAUTIONS

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

TECHNICAL GUIDELINES

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

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- After hydration, all standards and controls must be transferred to polypropylene tubes.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at ≤ -20°C for 1 month and at ≤ -80°C for greater than one month.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Bead Mix 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 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. Adjust probe height according to the protocols recommended by Luminex to the kit **filter plate** using **3** Alignment discs prior to reading an assay.
- When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit **solid plate** using **4** alignment discs. When reading the assay on FLEXMAP 3D™, adjust probe height according to the protocols recommended by Luminex to 0.74” for the kit solid plate.

- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in blank, standard curve and controls. If samples are diluted in assay buffer, use the assay buffer as matrix.
- 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 chunks. Centrifuge samples before use.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000xg. Remove serum and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Serum samples should be diluted 1:1 in the Assay Buffer provided in the kit (i.e. one part serum sample into one part Assay Buffer). When further dilution beyond 1:1 is required, use Serum Matrix as the diluent.

B. Preparation of Plasma Samples:

- Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1000xg within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Plasma samples should be diluted 1:1 in the Assay Buffer provided in the kit (i.e. one part plasma sample into one part Assay Buffer). When further dilution beyond 1:1 is required, use Serum Matrix as the diluent.

C. Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- Tissue culture supernatant may require a dilution with an appropriate control medium prior to assay.

NOTE:

- A maximum of 25 µL per well of diluted serum or plasma can be used. Tissue culture or other media may also be used.
- All samples must be stored in polypropylene tubes. **DO NOT STORE SAMPLES IN GLASS.**
- Avoid debris, lipids and cells when using samples with gross hemolysis or lipemia.
- Care must be taken when using heparin as an anticoagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

If premixed beads are used, sonicate the premixed bead bottle 30 seconds and then vortex for 1 minute before use.

For individual vials of beads, sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 60 µL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Assay Buffer Vortex the mixed beads well. Unused portion may be stored at 2-8°C for up to one month.

Example 1: When using 5 cytokine antibody-immobilized beads, add 60 µL from each of the 5 bead sets to the Mixing Bottle. Then add 2.7mL Assay Buffer.

Example 2: When using 9 cytokine antibody-immobilized beads, add 60 µL from each of the 9 bead sets to the Mixing Bottle. Then add 2.46 mL Assay Buffer .

B. Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 µL deionized water. Invert the vial several times to mix and vortex. Allow the vial to sit for 5-10 minutes and then transfer the controls to appropriately labeled polypropylene microfuge tubes. Unused portion may be stored at ≤ -20°C for up to one month.

C. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 30 mL of 10X Wash Buffer with 270 mL deionized water. Store unused portion at 2-8°C for up to one month.

D. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Add 2.0 mL Assay Buffer to the bottle containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution. Leftover reconstituted Serum Matrix should be stored at ≤ -20°C for up to one month.

E . Preparation of Mouse Cytokine Panel II Standard

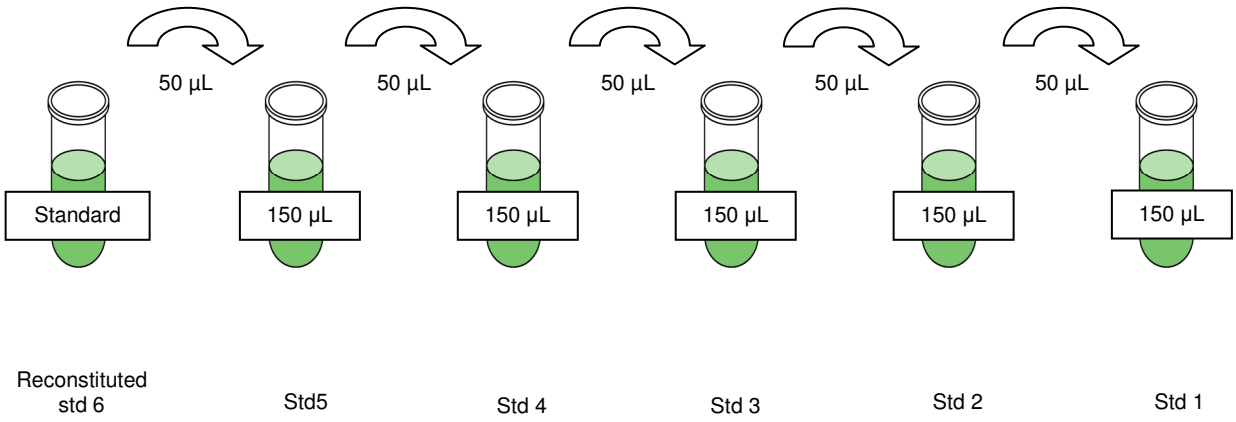
1.) Prior to use, reconstitute the Mouse Cytokine Standard with 250 µL deionized water to give 100,000 pg/mL (for Fractalkine, IL-25), 50,000 pg/mL (for EPO), and 10,000 pg/mL (for all other analytes). Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes and then transfer the standard to an appropriately labeled polypropylene microfuge tube. This will be used as the Std 6; the unused portion may be stored at ≤ -20°C for up to one month.

2). Preparation of Working Standards

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

Standard	Volume of Deionized Water to Add	Volume of Standard to Add
Original (Std 6)	250 µL	0

Standard	Volume of Assay Buffer to Add	Volume of Standard to Add
Standard 5	150 µL	50 µL of Standard 6
Standard 4	150 µL	50 µL of Standard 5
Standard 3	150 µL	50 µL of Standard 4
Standard 2	150 µL	50 µL of Standard 3
Standard 1	150 µL	50 µL of Standard 2



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

Standard Tube #	EPO (pg/mL)	Fractalkine, IL-25 (pg/mL)	All Other Analytes (pg/mL)
1	49	98	9.8
2	195	391	39
3	782	1563	156
4	3125	6250	625
5	12,500	25,000	2500
6	50,000	100,000	10,000

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), Std1, Std2, Std3, Std4, Std5, and Std6], 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. Prewet the plate by pipetting 200 µL of Wash Buffer into each well of the Microtiter Filter Plate. Seal and shake on a plate shaker for 10 minutes at room temperature (20-25°C).

2. Decant Wash Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.

3. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 pg/mL standard (Background).

4. Add 25 µL of Assay Buffer to the sample wells.

5. Add 25 µL of appropriate matrix solution to the background, standards, and control wells. When assaying serum or plasma, use the Serum Matrix provided in the kit. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.

6. Add 25 µL of 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. 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.)

8. Seal the plate with a plate sealer, cover it with the lid. Wrap a rubber band around the plate holder, plate and lid and incubate with agitation on a plate shaker overnight at 4°C or 2 hours at room temperature (20-25°C). *An overnight incubation (16-18 hr) may improve assay sensitivity for some analytes.*

Add 200 µL Wash Buffer per well

Shake 10 min, RT

Decant

- Add 25 µL Standard or Control to appropriate wells
- Add 25 µL Assay Buffer to background and sample wells

- Add 25 µL appropriate matrix to background, standards, and control wells
- Add 25 µL diluted Samples to sample wells
- Add 25 µL Beads to each well

Incubate overnight at 4℃ or at RT for 2 hours with shaking

9. Gently remove fluid by aspiration. **(NOTE: DO NOT INVERT PLATE.)** To avoid aspiration related bead loss, allow the plate to soak on the magnet of the plate washer for 60 seconds prior to aspiration.

Aspirate and wash 2X with 200 µL Wash Buffer

10. Wash plate 2 times with 200 µL/well of Wash Buffer, removing Wash Buffer by aspiration between each wash. To avoid washing/aspiration related bead loss, allow approximately 60 seconds between dispensing of the Wash Buffer and subsequent aspiration.

    Note: If using the recommended Plate washer for magnetic beads (Bio-Tek ELx405) follow the appropriate equipment settings outlined on Page 17.

11. Add 25 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)

Add 25 µL Detection Antibodies per well

12. Seal, cover with lid and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT ASPIRATE AFTER INCUBATION.**

Incubate 1 hour at RT

Do Not Aspirate

13. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.

Add 25 µL Streptavidin-Phycoerythrin per well

14. Seal, cover with lid and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).

Incubate for 30 minutes at RT

15. Gently remove all contents by aspiration. **(NOTE: DO NOT INVERT PLATE.)** To avoid aspiration related bead loss, allow the plate to soak on the magnet of the plate washer for 60 seconds prior to aspiration. Wash plate 2 times with 200 µL/well Wash Buffer, removing Wash Buffer by aspiration between each wash. To avoid washing/aspiration related bead loss, allow approximately 60 seconds between dispensing of the Wash Buffer and subsequent aspiration.

    Note: If using the recommended Plate washer for magnetic beads, (Bio-Tek ELx405) follow the appropriate equipment settings outlined on Page 17.

Aspirate and wash 2X with 200 µL Wash Buffer

16. Add 150 µL of Sheath Fluid to all wells. Resuspend the beads on a plate shaker for 5 minutes.
17. Run plate on Luminex $^{200™, HTS}$ or FLEXMAP 3D^{TM} with xPONENT software.
18.. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating cytokine/chemokines concentrations in samples. (Note: For diluted samples, multiply the calculated concentration by the dilution factor.)

Add 150 µL Sheath Fluid per well

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

EQUIPMENT SETTINGS

Bio-Tek ELx405:

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

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

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

2.) Wash program:

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

Dispense:
1. Dispense volume: 200 µL/well
2. Dispense flow rate: 5
3. Dispense height: 130 (16.51 mm)
4. Horizontal disp pos: 00 (0 mm)
5. Bottom Wash first?: NO
6. Prime before start?: NO

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

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

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

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

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

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

Events:	50, per bead	
Sample Size:	100 µL	
Gate Settings	8,000 to 15,000	
Time Out	60 seconds	
Bead Set:	Customizable 12-Plex	12-Plex Premix Beads
EPO	12	12
Exodus-2	14	14
MCP-5	18	18
MIP-3β	20	20
MIP-3a	22	22
TARC	27	27
IL-16	29	29
Fractakine	33	33
IL-21	35	35
IL-22	37	37
IL-25	39	39
IL-28B	42	42

QUALITY CONTROLS

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located at the Millipore website www.millipore.com/techlibrary/index.do using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Cross-Reactivity

There was no or negligible cross-reactivity between the antibodies and any of the other analytes in this panel.

Assay Sensitivities (minimum detectable concentrations, pg/mL)

MinDC: Minimum Detectable Concentration is calculated by the StatLIA® Immunoassay Analysis Software from Brendan Technologies. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions.

Cytokines	Overnight Protocol (N=7)
	MinDC +2SD (pg/mL)
EPO	22
Exodus-2	14
MCP-5	2
MIP-3β	20
MIP-3a	9
TARC	3
IL-16	30
Fractakine	120
IL-21	15
IL-22	2
IL-25	350
IL-28B	17

Precision

Intra-assay precision is generated from the mean of the % CV's from 8 reportable results across two different concentration of cytokines in a single assay. Inter-assay precision is generated from the mean of the % CV's from 4-8 reportable results across two different concentrations of cytokine across 6 different assays.

Cytokines	Overnight Protocol (N=6 assays)	
	Intra-assay %CV	Inter-assay %CV
EPO	2.6	10.9
Exodus-2	3.7	5.6
MCP-5	2.6	6.1
MIP-3β	1.4	9.8
MIP-3a	1.3	7.4
TARC	1.9	10.2
IL-16	2.1	7.5
Fractakine	2.8	14.2
IL-21	3.7	10.4
IL-22	2.3	10.1
IL-25	4.4	6.5
IL-28B	1.9	7.2

Accuracy

Defined as percent recovery, is generated from the mean of % recovery of 6 levels of cytokines spiked into matrix in 4 -6 independent experiments.

Cytokines	Overnight Protocol
	% recovery in matrix
EPO	99.7
Exodus-2	95.3
MCP-5	99.8
MIP-3β	98.1
TARC	100.2
MIP-3a	98.2
IL-16	93.7
Fractakine	99.8
IL-21	100.6
IL-22	103.5
IL-25	108.4
IL-28B	96.0

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Filter plate will not vacuum	Vacuum pressure is insufficient	Increase vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble particles	Centrifuge samples just prior to assay set-up and use supernatant. If high lipid concentration, after centrifugation, remove lipid layer and use supernatant.
	Sample too viscous	May need to dilute sample.
Insufficient bead count	Vacuum pressure too high	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds.
	Bead mix prepared incorrectly	Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting into the plate.
	Samples cause interference due to particulate matter or viscosity	See above. Also sample probe may need to be cleaned with alcohol flush, backflush and washes; or, if needed, probe should be removed and sonicated.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
Plate leaked	Vacuum pressure too high	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (prewetted) plate and continue.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate stand or raised edge so bottom of filter is not touching any surface.
	Insufficient blotting of filter plate bottom causing wicking	Blot the bottom of the filter plate well with absorbent towels after each wash step.
	Pipette touching plate filter during additions	Pipette to the side of well.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately and by pipeting with multichannel pipets without touching reagent in plate.
	Matrix used has endogenous analyte or interference	Check matrix ingredients for crossreacting components (e.g. interleukin modified tissue culture medium).
	Insufficient washes	Increase number of washes.

Beads not in region or gate	Luminex not calibrated correctly or recently	Calibrate Luminex based on instrument manufacturer's instructions at least once a week or if temperature has changed by >3°C.
	Gate settings not adjusted correctly	Some Luminex instruments (e.g. Bio-Plex) require different gate settings than those described in the kit protocol. Use instrument default settings.
	Wrong bead regions in protocol template	Check kit protocol for correct bead regions or analyte selection.
	Incorrect sample type used	Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required.
	Instrument not washed or primed	Prime the Luminex 4 times to eliminate air bubbles. Wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid.
	Beads were exposed to light	Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.
Signal for whole plate is same as background	Incorrect or no Detection Antibody was added	Add appropriate Detection Antibody and continue.
	Streptavidin-Phycoerythrin was not added	Add Streptavidin-Phycoerythrin according to protocol. If Detection Antibody has already been vacuumed out, sensitivity may be low.
Low signal for standard curve	Detection Antibody may have been vacuumed out prior to adding Streptavidin Phycoerythrin	May need to repeat assay if desired sensitivity not achieved.
	Incubations done at incorrect temperatures, timings or agitation	Assay conditions need to be checked.
Signals too high, standard curves are saturated	Calibration target value set too high	With some Luminex instruments (e.g. Bio-Plex) default target setting for RP1 calibrator is set at High PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with standard curve and samples	Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte	If below detectable levels, it may be possible to use higher sample volume. Check with tech support for appropriate protocol modifications.
	Samples contain analyte concentrations higher than highest standard point	Samples may require dilution and reanalysis for that particular analyte.
	Standard curve was saturated at higher end of curve	See above.

High variation in samples and/or standards	Multichannel pipet may not be calibrated	Calibrate pipets.
	Plate washing was not uniform	Confirm all reagents are vacuumed out completely in all wash steps.
	Samples may have high particulate matter or other interfering substances	See above.
	Plate agitation was insufficient	Plate should be agitated during all incubation steps using a vertical plate shaker at a speed where beads are in constant motion without splashing.
	Cross-well contamination	Check when reusing plate sealer that no reagent has touched sealer. Care should be taken when using same pipet tips that are used for reagent additions and that pipet tip does not touch reagent in plate.

REPLACEMENT REAGENTS	**Catalog #**
Mouse Cytokine Panel II Standard	MXM8073
Mouse Cytokine Panel II Quality Controls	MXM6073
Serum Matrix	MXMSM
Mouse Cytokine Panel II Detection Antibodies	MXM1073
Streptavidin-Phycoerythrin	L-SAPE3
Assay Buffer	L-MAB
Set of two 96-Well Plates with Sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Magnetic Beads

Cytokines	Bead #	Cat. #
Premix 12 plex	--	MP2PMX12-MAG
EPO	12	MEP0-MAG
Exodus-2	14	MEXDS2-MAG
MCP-5	18	MMCP5-MAG
MIP-3β	20	MMIP3B-MAG
MIP-3a	22	MMIP3A-MAG
TARC	27	MTARC-MAG
IL-16	29	MIL16-MAG
Fractalkine	33	MFKN-MAG
IL-21	35	MIL21-MAG
IL-22	37	MIL22-MAG
IL-25	39	MIL25-MAG
IL-28B	42	MIL28B-MAG

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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										