Mouse Th17 Magnetic Bead Panel

96-Well Plate Assay

Cat. # MTH17MAG-47K or MT17MAG47K-PX25

MILLIPLEX® MAP

Mouse Th17 MAGNETIC BEAD PANEL KIT
96-Well Plate Assay

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

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INTRODUCTION

CD4+T-helper cells are major players in adaptive immunity. They can be divided into the major subsets Th1, Th2, Th17 and Treg based on their expression profile of transcription factors and secreted cytokines. Th1 cells, induced by IL-12 and enhanced by IFNγ, secrete IFNγ for activating macrophages. Th2 cells, induced by IL-4, produce mainly IL-4, IL-5, IL-13, IL-25/IL-17E and IL-31 and play a role in allergy. Treg cells, induced by TGFβ, produce the anti-inflammatory cytokine IL-10 and also TGFβ and function to control T-cell responses to prevent autoimmune reactions. Th17 cells, a more recently discovered subset characterized by the production of IL-17, are induced by TGFβ, IL-6 and IL-21 in mice or TGFβ combined with IL-23 and IL-1β in humans. Activated Th17 cells secrete IL-17A, IL-17F, IL-6, IL-21, IL-22 and TNFα which promote tissue inflammation.

Th17 cells are involved in the clearance of extracellular bacteria and fungi. They are abundant in the intestinal lamina propria and function as a barrier against invading pathogens.

Excessive amounts of Th17 cells have been implicated in the pathogenesis of several autoimmune diseases such as multiple sclerosis, psoriasis, autoimmune uveitis, juvenile diabetes, rheumatoid arthritis, and Crohn's disease. In addition, Th17 cells play an important role in cancer development and progression, potentially playing a role in both promoting and inhibiting tumor growth.

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

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

Therefore, the **MILLIPLEX® MAP** Mouse Th17 Magnetic Bead panel enables you to focus on the therapeutic potential of the modulation of CD4+T-helper cell cytokines**.** Coupled with the Luminex xMAP® platform in a **magnetic bead** format, you receive the advantage of ideal speed and sensitivity, allowing quantitative multiplex detection of dozens of analytes simultaneously, which can dramatically improve productivity.

EMD Millipore's MILLIPLEX MOUSE TH17 Magnetic Bead panel is the most versatile system available for CD4+T-helper cell cytokine research.

- MILLIPLEX MAP offers you the ability to:
    - Select a 25-plex premixed kit or
    - Choose any combination of analytes from our panel of 25 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.

EMD Millipore's MILLIPLEX MAP Mouse Th17 Magnetic Bead kit is to be used for the simultaneous quantification of the following murine cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-15, IL-17A, IL-25/IL-17E, IL-17F, IL-21, IL-22, IL-23, IL-27, IL-28B, IL-31, IL-33, CD40L, GM-CSF, IFNγ, MIP-3α, TNFα, TNFβ.

This kit is for research purposes only. Not for use in Diagnostic Procedures.

Please read the entire protocol before use.

It is important to use same assay incubation conditions throughout your study.

PRINCIPLE

MILLIPLEX MAP is based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life-sciences and capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded magnetic beads known as MagPlex™-C microspheres.

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

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

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- 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 Th17 Standard	MTH17-8047	lyophilized	1 vial
Mouse Th17 Quality Controls 1 and 2	MTH17-6047	lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	MXMSM	lyophilized	1 vial
Set of one 96-Well Plate with 2 sealers	----------	----------	1 plate 2 sealers
Assay Buffer	L-AB	30 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	2 bottles
Mouse Th17 Detection Antibodies	MTH17-1047	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE3	3.2 mL	1 bottle
Mixing Bottle (not provided with premixed panel)	----------	----------	1 bottle

Mouse Th17 Antibody-Immobilized Premixed Magnetic Beads:

Premixed 25-plex Beads	MTH17PMX25-MAG	3.5 mL	1 bottle

Included Mouse Th17 Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel (see next page).

REAGENTS SUPPLIED (continued)

Mouse Th17 Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable 25 Analytes (50X concentration, 90µL)	
		Available	Cat. #
Anti-Mouse IL-25/IL-17E Bead	14	✓	MIL17E-MAG
Anti-Mouse GM-CSF Bead	15	✓	MGMCSF-MAG
Anti-Mouse IFNg Bead	19	✓	MIFNG-MAG
Anti-Mouse MIP-3a/CCL20 Bead	20	✓	MTHMIP3A-MAG
Anti-Mouse IL-1β Bead	25	✓	MIL1B-MAG
Anti-Mouse IL-2 Bead	26	✓	MIL2-MAG
Anti-Mouse IL-4 Bead	28	✓	MIL4-MAG
Anti-Mouse IL-5 Bead	30	✓	MIL5-MAG
Anti-Mouse IL-6 Bead	34	✓	MCYIL6-MAG
Anti-Mouse IL-21 Bead	35	✓	MIL21-MAG
Anti-Mouse IL-22 Bead	37	✓	MIL22-MAG
Anti-Mouse IL-28B Bead	42	✓	MIL28B-MAG
Anti-Mouse IL-10 Bead	43	✓	MIL10-MAG
Anti-Mouse IL-23 Bead	46	✓	MIL23-MAG
Anti-Mouse IL-12p70 Bead	47	✓	MIL12P70-MAG
Anti-Mouse IL-27 Bead	48	✓	MIL27-MAG
Anti-Mouse IL-13 Bead	52	✓	MIL13-MAG
Anti-Mouse IL-15 Bead	54	✓	MIL15-MAG
Anti-Mouse IL-17A Bead	56	✓	MIL17A-MAG
Anti-Mouse IL-17F Bead	61	✓	MIL17F-MAG
Anti-Mouse IL-33 Bead	67	✓	MIL33-MAG
Anti-Mouse IL-31 Bead	72	✓	MIL31-MAG
Anti-Mouse TNFβ Bead	73	✓	MTNFB-MAG
Anti-Mouse TNFa Bead	77	✓	MCYTNFA-MAG
Anti-Mouse CD40L Bead	78	✓	MCD40L-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 µL to 1000 µL
2. Multichannel Pipettes capable of delivering 5 µL to 50 µL or 25 µL to 200 µL
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. Rubber Bands
6. Aluminum Foil
7. Absorbent Pads
8. Laboratory Vortex Mixer
9. Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
10. Titer Plate Shaker (Lab-Line Instruments Model #4625 or equivalent)
11. Luminex 200™, HTS, FLEXMAP 3D™, or MAGPIX® with xPONENT software by Luminex Corporation
12. Automatic Plate washer for magnetic beads (Bio-Tek ELx405, EMD Millipore Catalog #40-015 or equivalent) or Hand-held Magnetic Separation Block (EMDMillipore Catalog #40-285 or equivalent)

Note: If a plate washer or hand held magnetic separation block for magnetic beads is not available, one can use a microtiter filter plate (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.
- After hydration, all standards and controls may be kept in their polypropylene tubes.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at ≤ -20°C for 1 month and at ≤ -80°C for greater than one month.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8°C for up to one month.
- During the preparation of the standard curve, make certain to mix the higher concentration well before making the next dilution. Use a new tip with each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some 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 using 4 alignment discs. When reading the assay on FLEXMAP 3D™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc. When reading the assay on MAGPIX, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 2 alignment discs.

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

SAMPLE COLLECTION AND STORAGE

A. Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000xg. Remove serum and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- It is recommended to use neat samples in the assay.

B. Preparation of Plasma Samples:

- Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1000xg within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- It is recommended to use neat samples in the assay.

C. Preparation of Tissue Culture Supernatant:

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

SAMPLE COLLECTION AND STORAGE (continued)

NOTE:

- A maximum of 25 µL per well of neat 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. (Note: Due to the composition of magnetic beads, you may notice a slight color in the bead solution. This does not affect the performance of the beads or the kit.)

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

Example 2: When using 10 antibody-immobilized beads, add 60 µL from each of the 10 bead vials to the Mixing Bottle. Then add 2.4 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 60 mL of 10X Wash Buffer (two bottles) with 540 mL deionized water. Store unused portion at 2-8°C for up to one month.

D. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Add 1.0mL 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 ≤ -20°C for up to one month**.**

E. Preparation of Mouse Th17 Standard

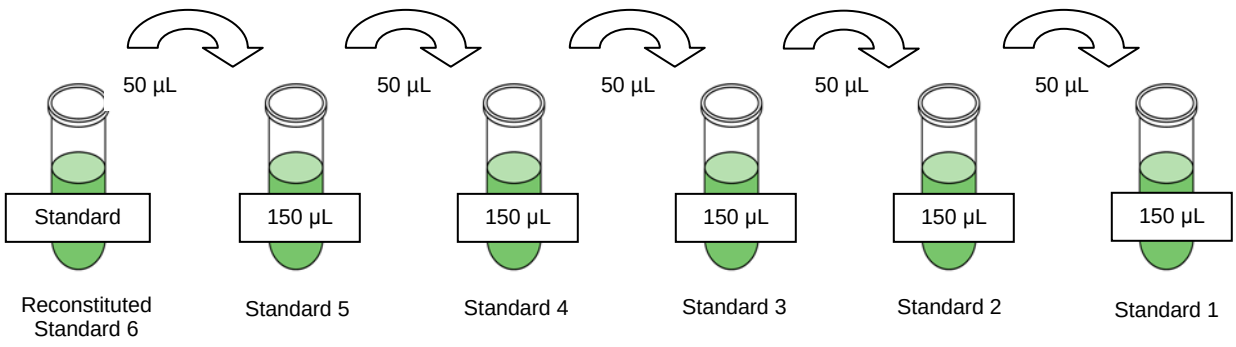
1.) Prior to use, reconstitute the Mouse Th17 Standard with 250 µL deionized water. Refer to table below for analyte concentrations. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes and then transfer the standard to an appropriately labeled polypropylene microfuge tube. This will be used as standard 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 as Standard 5, Standard 4, Standard 3, Standard 2 and Standard 1. Add 150 µL of Assay Buffer to each of the five tubes. Prepare 1:4 serial dilutions by adding 50 µL of the reconstituted Standard 6 to the Standard 5 tube, mix well and transfer 50 µL of the Standard 5 to the Standard 4 tube, mix well and transfer 50 µL of the Standard 4 to the Standard 3 tube, mix well and transfer 50 µL of the Standard 3 to the Standard 2 tube, mix well and transfer 50 µL of the Standard 2 to the Standard 1 tube. The 0 pg/mL Standard (Background) will be the Assay Buffer.

Standard	Volume of Deionized Water to Add	Volume of Standard to Add
Standard 6	250 µL	0

Standard Number	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



PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

Standard	IL-17E (pg/mL)	GM-CSF & IL-15 (pg/mL)	IFNg & IL-6 (pg/mL)	MIP-3a, IL-31 & CD40L (pg/mL)	IL-1β (pg/mL)	IL-2 (pg/mL)
Standard 1	586	34	7.8	49	15	6.9
Standard 2	2344	137	31.3	195	59	23.4
Standard 3	9375	547	125	781	234	94
Standard 4	37500	2188	500	3125	938	375
Standard 5	150000	8750	2000	12500	3750	1500
Standard 6	600000	35000	8000	50000	15000	6000

Standard	IL-4 (pg/mL)	IL-5 (pg/mL)	IL-21, IL-10 & IL-12p70 (pg/mL)	IL-22 (pg/mL)	IL-28B (pg/mL)	IL-23 (pg/mL)
Standard 1	1.5	4.9	20	2.4	127	342
Standard 2	5.9	19.5	78	9.8	508	1367
Standard 3	23	78	313	39	2031	5469
Standard 4	94	313	1250	156	8125	21875
Standard 5	375	1250	5000	625	32500	87500
Standard 6	1500	5000	20000	2500	130000	350000

Standard	IL-27 (pg/mL)	IL-13 & IL17A (pg/mL)	IL-17F (pg/mL)	IL-33 (pg/mL)	TNFβ (pg/mL)	TNFa (pg/mL)
Standard 1	879	39	10	78	488	3.4
Standard 2	3516	156	39	313	1953	13.7
Standard 3	14063	625	156	1250	7813	55
Standard 4	56250	2500	625	5000	31250	219
Standard 5	225000	10000	2500	20000	125000	875
Standard 6	900000	40000	10000	80000	500000	3500

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), Standards 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 Wash 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 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. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
6. Add 25 µL of sample (neat) 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.)

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 solution to background, Standards, and Control wells
- Add 25 µL neat samples to sample wells
- Add 25 µL Beads to each well

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. Alternatively, incubate for 2 hours at room temperature (20-25ºC), but the overall performance, including sensitivity may be less than for the overnight incubation.

9. Gently remove well contents and wash plate two 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 hr at room temperature (20-25°C). **DO NOT ASPIRATE AFTER INCUBATION.**

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

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

14. Gently Remove well contents and wash plate 2 times following instructions listed in the **PLATE WASHING** section.

15. Add 150 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells. Resuspend the beads in 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-fitting method for calculating analyte concentrations in samples. Note: For diluted samples, multiply the calculated concentration by the dilution factor.

Incubate overnight at 4°C or 2 hours at RT with shaking

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

Add 25 µL Detection Antibodies per well

Incubate 1 hr at RT (20-25°C)

Do Not Aspirate

Add 25 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

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

Add 150 µL Sheath Fluid or Drive Fluid per well

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

PLATE WASHING

1.) Solid Plate

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

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

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

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

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

EQUIPMENT SETTINGS

Bio-Tek ELx405:
The general recommended wash protocol (Link Protocol) is as follows:

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

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

2.) Wash program:

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

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

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

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

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

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

EQUIPMENT SETTINGS (continued)

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, 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 (EMD Millipore Cat #40-275) and performance verified with the Performance Verification Kit (EMD Millipore Cat #40-276). The Luminex FLEXMAP 3D™ instrument must be calibrated with the FLEXMAP 3D™ Calibrator Kit (EMD Millipore Cat #40-028) and performance verified with the FLEXMAP 3D™ Performance Verification Kit (EMD Millipore Cat #40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMDMillipore Cat #40-049) and performance verified with the MAGPIX® Performance Verification Kit (EMDMillipore Cat #40-050).

NOTE: These assays cannot be run on 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	
Reporter Gain:	Default (low PMT)	
Time Out:	60 seconds	
Bead Set:	Customizable 25-Plex Beads	
	IL-17E/IL-25	14
	GM-CSF	15
	IFNg	19
	MIP-3a/CCL20	20
	IL-1β	25
	IL-2	26
	IL-4	28
	IL-5	30
	IL-6	34
	IL-21	35
	IL-22	37
	IL-28B	42
	IL-10	43
	IL-23	46
	IL-12p70	47
	IL-27	48
	IL-13	52
	IL-15	54
	IL-17A	56
	IL-17F	61
	IL-33	67
	IL-31	72
	TNFβ	73
	TNFa	77
	CD40L	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 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 the other analytes in the panel when MXMSM matrix is added to the standard curve. When cell culture media with 10% FBS is added to the standard curve the assay may have ≤ 5% cross-reactivity between the TNFβ standard and the IL-17E, IL-2, IL-27, IL13 and IL-31 assays.

Assay Sensitivities (minimum detectable concentrations, pg/mL)

Minimum Detectable Concentration (MinDC) is calculated using the StatLia. 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=8)		2 Hour Protocol (n=3)	
	Min DC (pg/mL)	Min DC + 2 SD (pg/mL)	Min DC (pg/mL)	Min DC + 2 SD (pg/mL)
IL-17E/IL-25	357.3	475.3	425.7	530.6
GM-CSF	19.8	25.6	30.7	33.7
IFNg	1.5	2.2	2.4	3.3
MIP-3a/CCL20	9.4	11.8	12.7	13.8
IL-1β	3.3	4.2	4.7	7.0
IL-2	1.5	2.2	1.0	1.3
IL-4	0.4	0.5	0.4	0.5
IL-5	5.1	8.3	4.6	5.1
IL-6	3.3	4.7	4.8	5.4
IL-21	10.9	15.7	9.0	13.0
IL-22	0.5	0.6	0.5	0.6
IL-28B	29.3	35.9	27.3	34.9
IL-10	5.6	7.5	5.7	6.8
IL-23	70.4	103.4	80.7	103.7
IL-12p70	12.9	17.3	13.7	14.8
IL-27	498.6	755.1	740.0	1071.6
IL-13	32.1	40.2	39.0	61.3
IL-15	8.6	11.0	12.0	17.3
IL-17A	18.6	28.9	33.7	38.3
IL-17F	5.2	6.4	9.6	10.9
IL-33	14.9	19.5	33.7	42.9
IL-31	25.8	33.9	51.7	63.4
TNFβ	137.1	195.4	169.7	224.6
TNFa	1.3	1.8	1.6	2.1
CD40L	14.6	19.4	45.0	62.1

ASSAY CHARACTERISTICS (continued)

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	
	Intra-assay %CV	Inter-assay %CV
IL-17E/IL-25	3	8
GM-CSF	2	6
IFNg	2	6
MIP-3a/CCL20	3	7
IL-1β	2	4
IL-2	3	3
IL-4	3	5
IL-5	2	6
IL-6	3	6
IL-21	3	7
IL-22	3	5
IL-28B	3	9
IL-10	3	5
IL-23	2	10
IL-12p70	2	3
IL-27	4	9
IL-13	4	7
IL-15	4	5
IL-17A	3	6
IL-17F	3	5
IL-33	4	5
IL-31	4	5
TNFβ	4	12
TNFa	3	5
CD40L	4	6

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
	% Recovery in Serum Matrix
IL-17E/IL-25	110
GM-CSF	105
IFNg	97
MIP-3a/CCL20	108
IL-1β	103
IL-2	87
IL-4	96
IL-5	85
IL-6	100
IL-21	104
IL-22	101
IL-28B	106
IL-10	98
IL-23	101
IL-12p70	91
IL-27	101
IL-13	105
IL-15	102
IL-17A	92
IL-17F	102
IL-33	104
IL-31	99
TNFβ	101
TNFa	102
CD40L	103

TROUBLESHOOTING GUIDE

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

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

Problem	Probable Cause	Solution
	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 #**
Mouse Th17 Standard	MTH17-8047
Mouse Th17 Quality Controls 1 & 2	MTH17-6047
Serum Matrix	MXMSM
Mouse Th17 Detection Antibodies	MTH17-1047
Streptavidin-Phycoerythrin	L-SAPE3
Assay Buffer	L-AB
Set of two 96-Well plates with sealers	MAG-PLATE
10X Wash Buffer	L-WB

Mouse Th17 Antibody-Immoblized Premixed Magnetic Beads	
Premixed 25-plex Beads	MTH17PMX25-MAG

Antibody-Immobilized Magnetic Beads

Analyte	*Bead #*	*Cat. #*
IL-17E/IL-25	14	MIL17E-MAG
GM-CSF	15	MGMCSF-MAG
IFNg	19	MIFNG-MAG
MIP-3a/CCL20	20	MTHMIP3A-MAG
IL-1β	25	MIL1B-MAG
IL-2	26	MIL2-MAG
IL-4	28	MIL4-MAG
IL-5	30	MIL5-MAG
IL-6	34	MCYIL6-MAG
IL-21	35	MIL21-MAG
IL-22	37	MIL22-MAG
IL-28B	42	MIL28B-MAG
IL-10	43	MIL10-MAG
IL-23	46	MIL23-MAG
IL-12p70	47	MIL12P70-MAG
IL-27	48	MIL27-MAG
IL-13	52	MIL13-MAG
IL-15	54	MIL15-MAG
IL-17A	56	MIL17A-MAG
IL-17F	61	MIL17F-MAG
IL-33	67	MIL33-MAG
IL-31	72	MIL31-MAG
TNFβ	73	MTNFB-MAG
TNFa	77	MCYTNFA-MAG
CD40L	78	MCD40L-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										