User Guide

MILLIPLEX® 384-Well High Sensitivity Human T Cell Magnetic Bead Panel

384-Well Plate Assay

HSTC384–28K
HSTCMAG384-PX21
HSTCMAG384PX21BK

Introduction

Cytokines are immunomodulatory polypeptides that play key roles in both adaptive and innate immune responses. A generic term, "cytokines" includes myokines (produced by mononuclear phagocytic cells), lymphokines (produced by activated Th cells), interleukins (acting as mediators between T cells) and chemokines (responsible for T-cell migration). One of the regulatory mechanisms of the immune system, cytokines act at the recognition, activation, or effector phases of an immune response, modulating the development and functional activities of the subtypes of T cells, B cells and myeloid cells. Consequently, research involving cytokines plays a significant role in achieving a deeper understanding of the immune system and its multi-faceted response to most antigens, especially those responses that make up the inflammatory process.

Low levels of inflammation are involved in many clinical and sub-clinical disease states, such as autoimmune disease, cardiovascular disease, diabetes, neurological disorders and cancer. Measuring picogram levels of cytokines, therefore, is critical for understanding the pathogenesis of these diseases.

The MILLIPLEX® 384-Well High Sensitivity Human T Cell Magnetic Bead Panel enables you to focus on the therapeutic potential of cytokines, as well as the modulation of even low levels of cytokine expression. It is the most versatile system available for the study of low-level expression in cytokine research. The MILLIPLEX® portfolio offers you the ability to select a 21-plex pre-mixed kit or choose any combination of 21 analytes to design a custom kit that better meets your needs. In addition, data obtained from the High Sensitivity T Cell Panel will correlate with data for the respective cytokines in the Human Cytokine/Chemokine Panels I, II and III, furthering your ability to measure specific cytokine response in both normal and disease states.

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, dramatically improving productivity. While 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:

- Lower and more consistent CVs
- Improved performance with samples having high nonspecific binding
- More flexible plate/plate washer option

The MILLIPLEX® 384-Well High Sensitivity Human T Cell Magnetic Bead Panel is to be used for the simultaneous quantification of any or all of the following 21 human cytokines: Fractalkine, GM-CSF, IFNγ, IL-1β, IL–2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, IL-17A, IL–21, IL–23, ITAC, MIP-1α, MIP-1β, MIP-3α and TNFα in human plasma, serum, and cell/tissue culture supernatant samples. This panel provides biomedical researchers quality tools for the study of low-level inflammatory disease using a FLEXMAP 3D® or xMAP® INTELLIFLEX instrument.

For research use only. Not for use in diagnostic procedures.
Please read entire protocol before use.

Principle

MILLIPLEX® products are 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® products use 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.
- The following Luminex® instruments can be used to acquire and analyze data using two detection methods:
    - The Luminex® analyzers, Luminex® 200™, FLEXMAP 3D®, and xMAP® INTELLIFLEX, are 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. We combine 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.
- xMAP® INTELLIFLEX runs on INTELLIFLEX software for instrument control, run setup and generating high quality data with flexible output options. Data can be exported in xPONENT® style CSV files for compatibility with many existing analytical applications, or in the new, customizable INTELLIFLEX file format. The INTELLIFLEX file format is intended for flexibility and simplicity, allowing the user to freely select which data points to include and to reduce the time to analysis.

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

Store all reagents at 2–8 °C.

Reagents	Volume	Quantity	Cat. No.
Human High Sensitivity T Cell Standard	Lyophilized	1 vial	HSTC-8028
Human High Sensitivity T Cell Quality Controls 1 and 2	Lyophilized	2 vials	HSTC-6028
Serum Matrix*	Lyophilized	1 vial	MXHSM-7
Set of one 384-Well Plate with 2 sealers	-	1 set	-
Assay Buffer	15 mL	1 bottle	L-ABIR
10X Wash Buffer**	60 mL	1 bottle	L-WB
384-Well High Sensitivity Human T Cell Detection Antibodies	9 mL	1 bottle	HSTC384-1028
Streptavidin-Phycoerythrin	9 mL	1 bottle	MC-SAPE9
Bead Diluent	3.5 mL	2 bottles	LBD
Mixing Bottle (not provided with premixed panel)	-	1 bottle	-

* Contains 0.08% Sodium azide
** Contains 0.05% ProClin™

384-Well High Sensitivity Human T Cell Antibody-Immobilized Premixed Magnetic Beads:

	Volume	Quantity	Cat. No.
384-Well High Sensitivity Human T Cell Premixed 21-plex Beads	5 mL	1 bottle	HSTC384PX21-MAG

Included 384-Well High Sensitivity Human T Cell Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel.

384-Well High Sensitivity Human T Cell Antibody-Immobilized Magnetic Beads

Bead/Analyte Name	Luminex® Magnetic Bead Region	Customizable 21 Analytes (50X concentration, 125 µL) Available	Cat. No.	21-Plex Magnetic Premixed Beads
Anti-ITAC Beads	19	✔	HITAC-384MAG	✔
Anti-GM-CSF Beads	20	✔	HGMCSF-384MAG	✔
Anti-Fractalkine Beads	21	✔	HFKN-384MAG	✔
Anti-IFNγ Beads	25	✔	HIFNG-384MAG	✔
Anti-IL-10 Beads	27	✔	HIL10-384MAG	✔
Anti-MIP-3α Beads	28	✔	HMIP3A-384MAG	✔
Anti-IL-12 (p70) Beads	33	✔	HIL12P70-384MAG	✔
Anti-IL-13 Beads	35	✔	HIL13-384MAG	✔
Anti-IL-17A Beads	39	✔	HIL17-384MAG	✔
Anti-IL-1β Bead	46	✔	HIL1B-384MAG	✔
Anti-IL–2 Beads	48	✔	HIL2-384MAG	✔
Anti-IL–21 Beads	52	✔	HIL21-384MAG	✔
Anti-IL-4 Beads	53	✔	HIL4-384MAG	✔
Anti-IL–23 Beads	54	✔	HIL23-384MAG	✔
Anti-IL-5 Beads	55	✔	HIL5-384MAG	✔
Anti-IL-6 Beads	57	✔	HIL6-384MAG	✔
Anti-IL-7 Beads	61	✔	HIL7-384MAG	✔
Anti-IL-8 Beads	63	✔	HIL8-384MAG	✔
Anti-MIP-1α Beads	72	✔	HMIP1A-384MAG	✔
Anti-MIP-1β Beads	73	✔	HMIP1B-384MAG	✔
Anti-TNFα Beads	75	✔	HTNFA-384MAG	✔

Materials Required (Not included)

Reagents

MAGPIX® Drive Fluid PLUS (Cat. No. 40-50030), xMAP® Sheath Fluid PLUS (Cat. No. 40-50021), or xMAP® Sheath Concentrate PLUS (Cat. No. 40-50023)

Instrumentation/Materials

- Adjustable pipettes with tips capable of delivering 25 µL to 1000 µL
- Multichannel pipettes capable of delivering 5 µL to 50 µL, or 25 µL to 200 µL
- Reagent reservoirs
- Polypropylene microfuge tubes
- Rubber bands
- Aluminum foil
- Absorbent pads
- Laboratory vortex mixer
- Sonicator (Branson Ultrasonic Cleaner Model B200 or equivalent)
- Titer plate shaker (VWR® Microplate Shaker Cat. No. 12620-926 or equivalent)
- FLEXMAP 3D® with xPONENT® software or xMAP® INTELLIFLEX instrument with INTELLIFLEX software by Luminex® Corporation
- Automatic plate washer for magnetic beads (BioTek® Multiflo™ FX, Cat. No. 40-099 or equivalent) or Handheld Magnetic Separation Block (Cat. No. 40–290 or equivalent)
- Centrifuge with rotor for Microplates (Eppendorf 5810R or Sorvall Legend RT 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.

Symbol Definitions

Ingredient	Cat. No.	Label	
Human High Sensitivity T Cell Standard	HSTC-8028		**Danger.** Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/attention.
Human High Sensitivity T Cell Quality Controls 1 and 2	HSTC-6028		**Danger.** Harmful if swallowed. Causes serious eye damage. Harmful to aquatic life with long lasting effects. Avoid release to the environment. Wear eye protection. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Get medical advice/attention.
Serum Matrix	MXHSM-7		Harmful to aquatic life with long lasting effects. Avoid release to the environment**.**
384-Well High Sensitivity Human T Cell Detection Antibodies	HSTC384-1028		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Streptavidin-Phycoerythrin	MC-SAPE9		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
Assay Buffer	L-ABIR		**Warning.** Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
10X Wash Buffer	L-WB		**Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.

Unique Features

Please read this protocol with care as there are several distinctive steps as summarized below:

- When testing serum or plasma samples, the Standard and the Quality Control vials are reconstituted in Serum Matrix.
    - Both the reconstituted Quality Controls and the Standards are further diluted in Serum Matrix to make the final solutions.
    - Serum Matrix is reconstituted to a final volume of 4 mL.
    - Serum Matrix is used for the background wells.
- When testing tissue culture or other supernatant, the Quality Control and the Standard Vials should be reconstituted and further diluted in the appropriate control culture medium, which will also be used for the background wells.
- Add 40 µL background, Standard and Quality Controls to their appropriate wells on the assay plate.
- Add 20 µL Sample and 20 µL Assay Buffer to the sample wells resulting in a two-fold sample dilution.
- For Quality Control analysis, analyte concentrations DO NOT NEED to be multiplied by the dilution factor.
- Serum or plasma samples with high analyte values may be further diluted in serum matrix prior to the addition of 20 µL to the sample wells.
- It is recommended that the 384-well plate be centrifuged at 200 x *g* for 1 minute after loading reagents and before incubation with shaking, to ensure all reagents are in the bottom of the well.

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 ≤ –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 two-fold 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 the FLEXMAP 3D® instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 1 alignment disc.
- For the xMAP® INTELLIFLEX instrument, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.

- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background and for reconstitution of standard curve and controls.
- For serum/plasma samples that require dilution, use the MXHSM-7 provided and prepared as described in the kit for a two-fold dilution (for example, 50 µL of sample and 50 µL of MXHSM-7).
- 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

Preparation of Serum Samples

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000 x *g*. 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.
- Neat Serum samples are used. If further dilution is required, we recommend diluting samples no more than one to two in MXHSM-7 (for example, 50 µL sample and 50 µL MXHSM-7).

Preparation of Plasma Samples

- Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1000 x *g* 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.
- Neat Plasma samples are used. If further dilution is required, we recommend diluting samples no more than one to two in MXHSM-7 (for example, 50 µL sample and 50 µL MXHSM-7).

Preparation of Tissue Culture Supernatant

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

NOTE:

- A maximum of 20 µL per well of neat or of one to two 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 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

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 100 µL from each antibody-bead vial to the Mixing Bottle and bring final volume to 5.0 mL with LBD. 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.)

For example, when using 10 antibody-immobilized beads, add 100 µL from each of the 10 bead vials to the Mixing Bottle. Then add 4.0 mL LBD.

Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Add 1.0 mL deionized water to the bottle containing lyophilized serum matrix (Cat. No. MXHSM-7). Mix well. Allow at least 10 minutes for complete reconstitution. Add 3 mL Assay Buffer (Cat. No. L-ABIR) to the bottle for a final volume of 4 mL. Unused reconstituted matrix should be stored at ≤ –20 °C for up to one month.

Preparation of Quality Controls

For serum and plasma samples, reconstitute Quality Control 1 (QC1) and Quality Control 2 (QC2) vials with **250 µL MXHSM-7**. **These are the Stock QC Vials.** Invert the Stock Vials several times to mix and vortex. Allow the vials to sit for 5-10 minutes. Transfer the reconstituted Quality Control 1 and Quality Control 2 into two polypropylene microfuge tubes and set in an ice bath. Label two tubes QC1 and QC2 and add 150 µL MXHSM-7 to each tube. Remove 50 µL from QC1 or QC2 Stock Vials and add to the 150 µL MXHSM-7 in the respective QC1 and QC2 tubes and vortex and set in an ice bath. **Use these one to four diluted QCs in the assay**. These should be added to the plate within 1 hour of dilution. Unused portions of QC1 and QC2 stocks may be stored at ≤ –20 °C for up to one month.

For other samples (tissue culture, cell culture etc.) substitute the appropriate sample media for the MXHSM-7 used for serum and plasma samples above.

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 °C for up to one month.

Preparation of 384-Well High Sensitivity Human T Cell Standard

1. For serum and plasma samples, reconstitute the 384-Well High Sensitivity Human T Cell Standard with **250 µL MXHSM-7**. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. Transfer the reconstituted standard to a polypropylene microfuge tube. **This is the Stock Standard Vial NOT Standard 7**. Unused Stock Standard may be stored at ≤ –20 °C for up to one month. This reconstituted standard and the serially diluted standards in the following steps should be set in an ice bath, during the assay procedure. These need to be added to the plate within 1 hour of preparation.

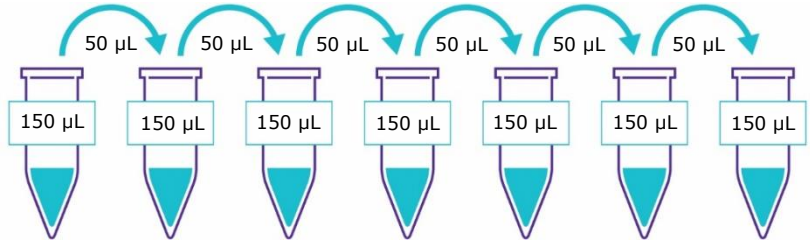
2. Preparation of Working Standards
   For serum and plasma samples, label seven polypropylene microfuge tubes as Standard 7, Standard 6, Standard 5, Standard 4, Standard 3, Standard 2 and Standard 1. Add 150 µL of MXHSM-7 to each of the seven tubes. Prepare serial dilutions by adding 50 µL of the Stock Standard to the Standard 7 tube, mix well and transfer 50 µL of the Standard 7 to the Standard 6 tube, mix well and transfer 50 µL of the Standard 6 tube to the Standard 5 tube, mix well and transfer 50 µL of the Standard 5 tube to the Standard 4 tube, mix well and transfer 50 µL of the Standard 4 tube to the Standard 3 tube, mix well and transfer 50 µL of the Standard 3 tube to the Standard 2 tube, mix well and transfer 50 µL of the Standard 2 tube to the Standard 1 tube and mix well. The 0 pg/mL standard (Background) will be MXHSM-7 or appropriate sample media.

Standard No.	Add MXHSM-7 (µL)	Add Standard (volume)
Stock Standard	250	0

Standard No.	Add MXHSM-7 (µL)	Add Standard (volume)
Standard 7	150	50 µL of Stock Standard
Standard 6	150	50 µL of Standard 7
Standard 5	150	50 µL of Standard 6
Standard 4	150	50 µL of Standard 5
Standard 3	150	50 µL of Standard 4
Standard 2	150	50 µL of Standard 3
Standard 1	150	50 µL of Standard 2

3. For other samples (tissue culture, cell culture etc.), substitute the appropriate media for the MXHSM-7 used for serum and plasma samples above.

Preparation of Standards



Standard 7 150 µL MXHSM-7 and 50 µL Stock Standard | Standard 6 | Standard 5 | Standard 4 | Standard 3 | Standard 2 | Standard 1

Standard	ITAC, IL-10 (pg/mL)	GM-CSF (pg/mL)	Fractalkine (pg/mL)	IFNγ, MIP-3α (pg/mL)
Standard 1	1.46	1.22	18.3	0.61
Standard 2	5.86	4.88	73.2	2.44
Standard 3	23.4	19.5	293	9.8
Standard 4	93.8	78.1	1,171.9	39
Standard 5	375	312.5	4,687.5	156
Standard 6	1,500	1,250	18,750	625
Standard 7	6,000	5,000	75,000	2,500

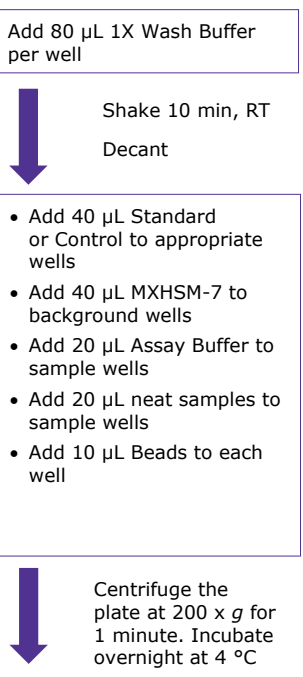
Standard	IL-12p70, IL-1β, IL–2, IL-5 (pg/mL)	IL-13, IL–21 (pg/mL)	IL-17A (pg/mL)	IL-4 (pg/mL)	IL–23 (pg/mL)
Standard 1	0.49	0.24	0.73	1.83	7.93
Standard 2	1.95	0.98	2.93	7.32	31.7
Standard 3	7.81	3.91	11.7	29.3	127
Standard 4	31.3	15.63	46.9	117.2	507.8
Standard 5	125	62.5	187.5	468.8	2,031.3
Standard 6	500	250	750	1,875	8,125
Standard 7	2,000	1,000	3,000	7,500	32,500

Standard	IL-6 (pg/mL)	IL-7 (pg/mL)	IL-8, MIP-1α (pg/mL)	MIP-1β (pg/mL)	TNFα (pg/mL)
Standard 1	0.18	0.37	0.31	0.92	0.43
Standard 2	0.73	1.46	1.22	3.66	1.71
Standard 3	2.93	5.86	4.88	14.7	6.84
Standard 4	11.7	23.4	19.5	58.6	27.3
Standard 5	46.9	93.8	78.1	234.4	109.4
Standard 6	187.5	375	312.5	937.5	437.5
Standard 7	750	1,500	1,250	3,750	1,750

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 7], 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 80 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 40 µL of each diluted Standard or Quality Control into the appropriate wells (NOT from Stock Vials). The Serum Matrix should be used for 0 pg/mL standard (background). When assaying tissue culture or other supernatant, use appropriate control culture medium as the background.
4. Add 20 µL of Assay Buffer to the sample wells.
5. Add 20 µL of sample into the sample wells.
6. Vortex Mixing Bottle and add 10 µL of the Mixed or Premixed Beads to each well. (**Note:** During addition of Beads, shake bead bottle intermittently to avoid settling.)
7. Seal the plate with a plate sealer. Centrifuge the plate at 200 x *g* for 1 minute. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-18 hrs) at 4 °C.



8. Gently remove well contents and wash plate 3 times following instructions listed in the Plate Washing section.
9. Add 20 µL of Detection Antibodies into each well.
(**Note:** Allow the Detection Antibodies to warm to room temperature prior to addition.)
10. Seal, cover with foil and centrifuge the plate at 200 x *g* for 1 minute. Incubate with agitation on a plate shaker for 1 hour at room temperature (20–25 °C). DO NOT ASPIRATE AFTER INCUBATION.
11. Add 20 µL Streptavidin-Phycoerythrin to each well containing the 20 µL of Detection Antibodies.
12. Seal, cover with foil and centrifuge the plate at 200 x *g* for 1 minute. Incubate with agitation on a plate shaker for 30 minutes at room temperature (20–25 °C).
13. Gently remove well contents and wash plate 3 times following instructions listed in the Plate Washing section.
14. Add 70 µL of Sheath Fluid PLUS to all wells. Resuspend the beads on a plate shaker for 5 minutes.
15. Run plate on a FLEXMAP 3D® instrument with xPONENT® software or xMAP® INTELLIFLEX instrument with INTELLIFLEX software.
16. 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 and Controls.
(**Note:** Because of the built-in two-fold sample dilution, for all neat samples, multiply the calculated concentrations by two. For two-fold diluted samples, multiply the calculated concentrations by four. Calculated Quality Control concentrations do not require multiplication by a dilution factor.)

Remove well contents and wash 3X with 80 µL Wash Buffer

Add 20 µL Detection Antibodies per well

Centrifuge then incubate for 1 hour at RT

Do Not Aspirate

Add 20 µL Streptavidin-Phycoerythrin per well

Centrifuge then incubate for 30 minutes at RT

Remove well contents and wash 3X with 80 µL Wash Buffer

Add 70 µL Sheath Fluid PLUS or Drive Fluid PLUS per well

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

Plate Washing

Using a magnetic plate washer.

Magnetic plate washer (Cat. No. 40-099)
Refer to specific automatic plate washer manual for appropriate equipment settings. Please note that after the final aspiration, there will be a small volume 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® Multiflo™ FX, please refer to the manufacturer's recommendations for programming instructions.

Equipment Settings

FLEXMAP 3D® instrument with xPONENT® software and xMAP® INTELLIFLEX instrument with INTELLIFLEX software:

These specifications are for the above listed instruments and software. Luminex® instruments with other software (for example, 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, each instrument must be calibrated and performance verified with the indicated calibration and verification kits.

Instrument	Calibration Kit	Verification Kit
FLEXMAP 3D®	FLEXMAP 3D® Calibrator Kit (Cat. No. F3D-CAL-K25)	FLEXMAP 3D® Performance Verification Kit (Cat. No. F3D-PVER-K25)
xMAP® INTELLIFLEX	xMAP® INTELLIFLEX Calibration Kit (Cat. No. IFX-CAL-K20)	xMAP® INTELLIFLEX Performance Verification Kit (Cat. No. IFX-PVER-K20)

NOTES

- When setting up a Protocol using the xPONENT® software, you must select MagPlex® as the Bead Type in the Acquisition settings.
- This assay can only be run on FLEXMAP 3D® or xMAP® INTELLIFLEX instruments.
- This assay 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. No. MAG384-PLATE, if additional plates are required for this purpose.

Events	50, per bead	
Sample Size	50 µL	
Gate Settings	8,000 to 15,000	
Reporter Gain	Default (low PMT)	
Time Out	60 seconds	
Bead Set	Customizable 21-Plex Beads	
	ITAC	19
	GM-CSF	20
	Fractalkine	21
	IFNγ	25
	IL-10	27
	MIP-3α	28
	IL-12 (p70)	33
	IL-13	35
	IL-17A	39
	IL-1β	46
	IL–2	48
	IL–21	52
	IL-4	53
	IL–23	54
	IL-5	55
	IL-6	57
	IL-7	61
	IL-8	63
	MIP-1α	72
	MIP-1β	73
	TNFα	75

Quality Controls

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located at our website SigmaAldrich.com using the catalogue 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 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.

	Overnight Protocol (n = 12 Assays)	
Analyte	**MinDC (pg/mL)**	**MinDC+2SD (pg/mL)**
ITAC	1.06	1.79
GM-CSF	0.86	1.68
Fractalkine	13.02	21.44
IFNγ	0.37	0.69
IL-10	0.98	1.75
MIP-3α	0.70	1.98
IL-12 (p70)	0.27	0.51
IL-13	0.16	0.30
IL-17A	0.57	1.15
IL-1β	0.32	0.55
IL–2	0.26	0.52
IL–21	0.19	0.33
IL-4	1.49	2.68
IL–23	6.91	13.73
IL-5	0.31	0.52
IL-6	0.14	0.24
IL-7	0.38	1.11
IL-8	0.16	0.31
MIP-1α	1.86	4.45
MIP-1β	0.71	1.44
TNFα	0.24	0.50

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 11 different assays.

	Overnight Protocol	
Analyte	**Intra-assay %CV**	**Inter-assay %CV**
ITAC	7.5	18.6
GM-CSF	2.7	20.8
Fractalkine	5.6	22.7
IFNγ	4.7	17.9
IL-10	6.3	20.2
MIP-3α	4.4	21.7
IL-12 (p70)	6.4	19.2
IL-13	4.6	19.2
IL-17A	8.1	18.9
IL-1β	4.5	17.3
IL–2	4.0	21.0
IL–21	5.7	17.7
IL-4	5.9	17.1
IL–23	2.9	19.3
IL-5	7.6	21.2
IL-6	6.1	19.2
IL-7	5.7	19.8
IL-8	5.0	20.2
MIP-1α	3.4	16.7
MIP-1β	2.6	18.2
TNFα	7.1	20.0

Accuracy

Spike Recovery: The data represent mean percent recovery of spiked standards ranging from low, medium, and high concentration in serum matrices (n=3).

	Overnight Protocol
Analyte	**% Recovery in Serum Matrix**
ITAC	94
GM-CSF	94
Fractalkine	108
IFNγ	95
IL-10	93
MIP-3α	93
IL-12 (p70)	101
IL-13	89
IL-17A	94
IL-1β	94
IL–2	91
IL–21	94
IL-4	90
IL–23	90
IL-5	88
IL-6	79
IL-7	83
IL-8	91
MIP-1α	95
MIP-1β	96
TNFα	91

Troubleshooting

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 flushes, back flushes and washes; or if needed, probe should be removed and sonicated.
	Probe height not adjusted correctly	When reading the assay on the FLEXMAP 3D® instrument, adjust probe height to the kit solid plate using 1 alignment disc. For the FLEXMAP 3D® instrument, when using the solid plate in the kit, the final resuspension should be with 80 µL Sheath Fluid PLUS in each well and 50 µL should be aspirated. When reading the assay on the xMAP® INTELLIFLEX instrument, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately and pipetting with multichannel pipettes without touching reagent in plate.
	Matrix used has endogenous analyte or interference	Check matrix ingredients for cross-reacting components (for example, interleukin modified tissue culture medium).
	Insufficient washes	Increase number of washes.

Problem	Probable Cause	Solution
Beads not in region or gate	Luminex® instrument not calibrated correctly or recently	Calibrate Luminex® instrument based on manufacturer's instructions, at least once a week or if temperature has changed by > 3 °C.
	Gate settings not adjusted correctly	Some Luminex® instruments (for example, 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® 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.
	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.

Problem	Probable Cause	Solution
Signals too high, standard curves are saturated	Calibration target value set too high	With some Luminex® instruments (for example, 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 technical 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 pipette may not be calibrated	Calibrate pipettes.
	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 an orbital 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 pipette tips that are used for reagent additions and that pipette tip does not touch reagent in plate.

Product Ordering

Replacement Reagents	Cat. No.
Human High Sensitivity T Cell Standard	HSTC-8028
Human High Sensitivity T Cell Quality Controls 1 and 2	HSTC-6028
Serum Matrix	MXHSM-7
384-Well High Sensitivity Human T Cell Detection Antibodies	HSTC384-1028
Streptavidin-Phycoerythrin	MC-SAPE9
Assay Buffer	L-ABIR
Set of two 384-Well plates with sealers	MAG384-PLATE
Bead Diluent	LBD
10X Wash Buffer	L-WB
384-Well Human High Sensitivity T Cell Panel 21 Plex Premixed Magnetic Bead Panel –BULK PACKAGED	HSTC384MAGPX21BK

Antibody-Immobilized Magnetic Beads

Analyte	Bead No.	Cat. No.
ITAC	19	HITAC-384MAG
GM-CSF	20	HGMCSF-384MAG
Fractalkine	21	HFKN-384MAG
IFNγ	25	HIFNG-384MAG
IL-10	27	HIL10-384MAG
MIP-3α	28	HMIP3A-384MAG
IL-12 (p70)	33	HIL12P70-384MAG
IL-13	35	HIL13-384MAG
IL-17A	39	HIL17-384MAG
IL-1β	46	HIL1B-384MAG
IL–2	48	HIL2-384MAG
IL–21	52	HIL21-384MAG
IL-4	53	HIL4-384MAG
IL–23	54	HIL23-384MAG
IL-5	55	HIL5-384MAG
IL-6	57	HIL6-384MAG
IL-7	61	HIL7-384MAG
IL-8	63	HIL8-384MAG
MIP-1α	72	HMIP1A-384MAG
MIP-1β	73	HMIP1B-384MAG
TNFα	75	HTNFA-384MAG
Premixed 21-plex Beads		HSTC384PX21-MAG

Well Map

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	0 Standard	QC No. 1																						
B	0 Standard	QC No. 1																						
C	Standard No. 1	QC No. 2																						
D	Standard No. 1	QC No. 2																						
E	Standard No. 2	Sample 1																						
F	Standard No. 2	Sample 1																						
G	Standard No. 3	Sample 2																						
H	Standard No. 3	Sample 2																						
I	Standard No. 4	Etc.																						
J	Standard No. 4																							
K	Standard No. 5																							
L	Standard No. 5																							
M	Standard No. 6																							
N	Standard No. 6																							
O	Standard No. 7																							
P	Standard No. 7																							

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