PRINCIPLE OF THE LUMINEX MULTIPLEX BEAD IMMUNOASSAY METHOD

Luminex Multiplex Bead Immunoassays are solid phase sandwich immunoassays, which are designed to be analyzed with a Luminex 100/200™ instrument. The spectral properties of 100 distinct bead regions can be monitored with the Luminex 100/200™ instrument, a capability that affords this assay system the potential for measuring up to 100 different analytes in a single sample. Beads of defined spectral properties conjugated to analyte specific capture antibodies and samples (including standards of known analyte concentration, control specimens, and unknowns) are pipetted into the wells of a filter bottom microplate and incubated. During this first incubation, analytes bind to the capture antibodies on the beads. After washing the beads, analyte-specific biotinylated detector antibodies are added and incubated with the beads. During this second incubation, the analyte-specific biotinylated detector antibodies recognize their epitopes and bind to the appropriate immobilized analytes. After removal of excess biotinylated detector antibodies, streptavidin conjugated to the fluorescent protein, R-Phycoerythrin (Streptavidin-RPE), is added and incubated. During this final incubation, the Streptavidin-RPE binds to the biotinylated detector antibodies associated with the immune complexes on the beads, forming a four-member solid phase sandwich. After washing to remove unbound Streptavidin-RPE, the beads are analyzed with the Luminex 100/200™ instrument. By monitoring the spectral properties of the beads and the amount of associated R-Phycoerythrin (RPE) fluorescence, the concentration of one or more analytes can be determined.

PRINCIPLE OF THE R&D ASSAY

Fluorokine_ MAP cytokine multiplex kits are designed for use with the Luminex 100™, Luminex 200™, or BioRad BioPlex™ dual laser, flow-based sorting and detection analyzers manufactured by Luminex Corporation. Analyte-specific antibodies are pre-coated onto color-coded microparticles. Microparticles, standards and samples are pipetted into wells. During an incubation period, the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the biotinylated detection antibodies, is added to each well. A final wash removes unbound Streptavidin-PE and the microparticles are resuspended in buffer and read using the Luminex analyzer. One laser is microparticle-specific and determines which analyte is being detected. The other laser determines the magnitude of the phycoerythrin-derived signal, which is in direct proportion to the amount of analyte bound.

Human Adipokine Magnetic Bead Panel 2

96-Well Plate Assay

Cat. # HADK2MAG-61K

MILLIPLEX® MAG

HUMAN ADIPOKINE MAGNETIC BEAD PANEL 2
96-Well Plate Assay

HADK2MAG-61K

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

Human Adipokine Magnetic Bead Panel 2

INTRODUCTION

In the past, the role of white adipose tissue was thought to be limited to energy storage and internal organ protection. The discovery of leptin secretion from adipocytes in 1994 led to the recognition that white adipose tissue is involved in a variety of metabolic and physiological processes. Adipocytes secrete a number of hormones called adipokines with functions that include appetite and energy balance, insulin sensitivity and lipid metabolism. One of these adipokines, adiponectin, is involved in the regulation of lipid and glucose metabolism, influencing the body's response to insulin. Also, its anti-inflammatory effects on the cellular lining of blood vessel walls may help explain the association of high adiponectin levels with the reduced risk of heart attack.

MILLIPLEX® MAP offers the broadest selection of analytes across a wide range of disease states and species. Once the analytes of interest have been identified, you can rely on the quality that we build into each kit to produce results you can trust. In addition to the assay characteristics listed in the protocol, other performance criteria evaluated during the validation process include: cross-reactivity, dilution linearity, kit stability, and sample behavior (e.g. detectability and stability).

Each MILLIPLEX® MAP panel and kit includes:

- Quality controls (QCs) provided to qualify assay performance
- Comparison of standard (calibrator) and QC lots to a reference lot to ensure lot-to-lot consistency
- Optimized serum matrix to mimic native analyte environment
- Detection antibody cocktails designed to yield consistent analyte profiles within panel

In addition each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The MILLIPLEX® MAP Human Adipokine Magnetic Bead Panel 2 thus enables you to focus on the therapeutic potential of adipokines. 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® MAP Human Adipokine Magnetic Bead Panel 2 Kit is part of the most versatile system available for obesity-related disorders of metabolic research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically validate and build the most comprehensive library available for protein detection and quantitation.

EMD Millipore's MILLIPLEX MAG Human Adipokine Magnetic Bead Panel 2 is the most versatile system available for obesity-related disorders of metabolic research.

- MILLIPLEX MAG offers you:
    - Choose any combination of analytes from our panel of 9 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 MAG Human Adipokine Magnetic Bead Panel 2 kit is a 9-plex kit to be used for the simultaneous quantification of any or all of the following analytes in human plasma or serum samples: HGF, IL-1β, IL-6, IL-8, Insulin, Leptin, MCP-1, NGF and TNFα.

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

Please read entire protocol before use.

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

PRINCIPLE

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

- Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- EMD Millipore provides three Luminex instruments to acquire and analyze data using two detection methods:
    - The Luminex analyzers Luminex 200™ and FLEXMAP 3D®, flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
    - The Luminex analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. EMD Millipore combines the streamlined data acquisition power of Luminex xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex instruments.

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

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2-8°C.
- For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze/thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

Reagents Supplied	Catalog Number	Volume	Quantity
Human Adipokine Panel 2 Standard	HADK2-8061-2	lyophilized	1 vial
Human Adipokine Panel 2 Quality Controls 1 and 2	HADK2-6061-2	lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	LHED-SD	lyophilized	1 vial
Set of one 96-Well Plate with 2 sealers	----------	----------	1 plate 2 sealers
Assay Buffer	LE-ABGLP	30 mL	1 bottle
Bead Diluent	LHE-BD	3.5 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	60 mL	1 bottle
Human Adipokine Panel 2 Detection Antibodies	HADK2-1061-2	5.5 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE7	5.5 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

Included Human Adipokine Magnetic Bead Panel 2 Antibody-Immobilized Magnetic Beads are dependent on customizable selection of analytes within the panel.

Human Adipokine Panel 2 Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable 9 Analytes (20X concentration, 200 µL)	
		Available	Cat. #
Anti-Human NGF Bead	20	✓	HNGF-MAG
Anti-Human IL-6 Bead	34	✓	HIL6-MAG
Anti-Human Insulin Bead	36	✓	HINS-MAG
Anti-Human Leptin Bead	39	✓	HLPTN-MAG
Anti-Human IL-8 Bead	44	✓	HIL8-MAG
Anti-Human HGF Bead	45	✓	HHGF-MAG
Anti-Human MCP-1 Bead	52	✓	HMCP1-MAG
Anti-Human TNFα	55	✓	HTNFA-MAG
Anti-Human IL-1β Bead	72	✓	HIL1B-MAG

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (EMD Millipore Catalog #SHEATHFLUID) or Luminex Drive Fluid (EMD Millipore 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 (BioTek® 405 LS and 405 TS, EMD Millipore Catalog #40-094, #40-095, #40-096, #40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog #40-285 or equivalent).

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

SAFETY PRECAUTIONS

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Note: See Full Labels of Hazardous components on next page.

Full labels of hazardous components in this kit:

Ingredient, Cat #		Full Label	
Human Adipokine Panel 2 Quality Controls 1 and 2	HADK2-6061-2		**Warning.** Harmful if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment.
Human Adipokine Panel 2 Standard	HADK2-8061-2		**Warning.** Harmful if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment.
Serum Diluent	LHED-SD		Harmful to aquatic life with long lasting effects. Avoid release to the environment.
Streptavidin-Phycoerythrin	L-SAPE7		**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.
Wash Buffer	L-WB		**Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.

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 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 or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc.

  For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.

TECHNICAL GUIDELINES (continued)

- For serum/plasma samples that require dilution use the Assay Buffer provided in the kit. The Serum Matrix will also require the same dilution with Assay Buffer. For example, if samples are diluted 1:2 in Assay Buffer, the Serum Matrix will need to be diluted 1:2 with Assay Buffer.
- 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.

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.

NOTE:

- A maximum of 25 µL per well of neat serum or plasma can 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

Sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 150 µL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent. 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 9 antibody-immobilized beads, add 150 µL from each of the 9 bead vials to the Mixing Bottle. Then add 1.65 mL Bead Diluent.

Example 2: When using 5 antibody-immobilized beads, add 150 µL from each of the 5 bead vials to the Mixing Bottle. Then add 2.25 mL Bead Diluent.

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 with 540 mL deionized water. Store unused portion at 2-8°C for up to one month.

D. Preparation of Serum Matrix

Add 1.0 mL deionized water to the bottle containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution. Leftover reconstituted Serum Matrix should be stored at ≤ -20°C for up to one month.

If serum/plasma samples require dilution, the Serum Matrix will also require the same dilution with Assay Buffer. For example, if samples are diluted 1:2 in Assay Buffer, the Serum Matrix will need to be diluted 1:2 with Assay Buffer.

E. Preparation of Human Adipokine Panel 2 Standard

1.) Prior to use, reconstitute the Human Adipokine Panel 2 Standard with 250 µL deionized water. 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 labeled Standard 7. The unused portion may be stored at ≤ -20°C for up to month.

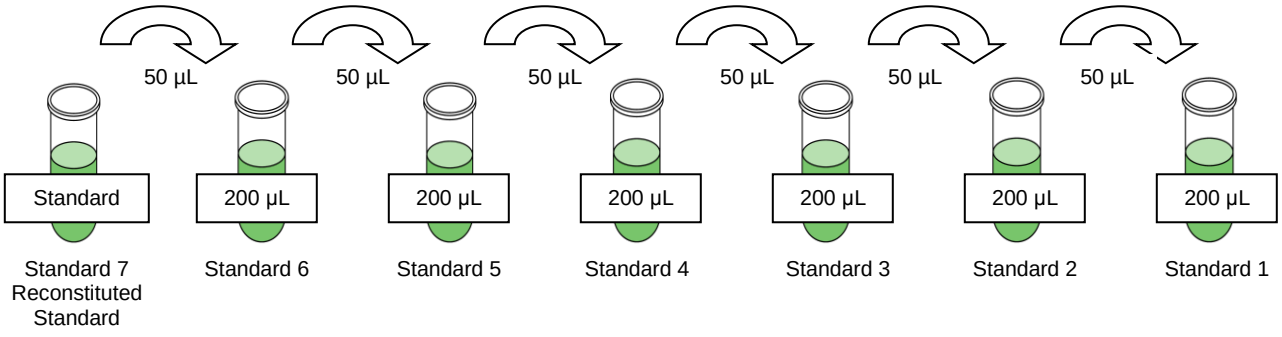
2). Preparation of Working Standards

Label 6 polypropylene microfuge tubes Standard 6, Standard 5, Standard 4, Standard 3, Standard 2 and Standard 1. Add 200 µL of Assay Buffer to each of the 6 tubes. Prepare serial dilutions by adding 50 µL of the Standard 7 reconstituted standard to the Standard 6 tube, mix well and transfer 50 µL of the 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 and mix well. The 0 pg/mL standard (Background) will be Assay Buffer.

Standard Tube	Volume of Deionized Water to Add	Volume of Standard to Add
Standard 7	250 µL	0

Standard Tube	Volume of Assay Buffer to Add	Volume of Standard to Add
Standard 6	200 µL	50 µL of Standard 7
Standard 5	200 µL	50 µL of Standard 6
Standard 4	200 µL	50 µL of Standard 5
Standard 3	200 µL	50 µL of Standard 4
Standard 2	200 µL	50 µL of Standard 3
Standard 1	200 µL	50 µL of Standard 2

Preparation of Standards



PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

Standard Concentrations

Standard Tube	IL-8, NGF, TNFα (pg/mL)	IL-6 (pg/mL)	IL-1β, MCP-1 (pg/mL)	HGF, Insulin (pg/mL)	Leptin (pg/mL)
Standard 1	0.64	0.96	1.3	9.6	38
Standard 2	3.2	4.8	6.4	48	192
Standard 3	16	24	32	240	960
Standard 4	80	120	160	1,200	4,800
Standard 5	400	600	800	6,000	24,000
Standard 6	2,000	3,000	4,000	30,000	120,000
Standard 7	10,000	15,000	20,000	150,000	600,000

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), 1, 2, 3, 4, 5, 6, 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 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 pg/mL standard (Background).
4. Add 25 µL of Assay Buffer to the sample wells.
5. Add 25 µL of Serum Matrix to the background, standards, and control wells.
6. Add 25 µL of Sample (neat) into the appropriate wells.
7. Vortex Mixing Bottle and add 25 µL of the Mixed to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-18 hours) at 4°C.

Add 200 µL Assay Buffer per well

↓ Shake 10 min, RT

↓ Decant

- Add 25 µL Standard or Control to appropriate wells
- Add 25 µL Assay Buffer to background and sample wells
- Add 25 µL Serum Matrix to background, standards, and control wells
- Add 25 µL Samples to sample wells
- Add 25 µL Beads to each well

↓ Incubate overnight (16-18 hours) at 4°C

9. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.

10. Add 50 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)

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

12. Add 50 µL Streptavidin-Phycoerythrin to each well containing the 50 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 3 times following instructions listed in the **PLATE WASHING** section.

15. Add 100 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 5 minutes.

16. Run plate on Luminex 200™, HTS, FLEXMAP 3D™ or MAGPIX® with xPONENT software.

17. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. Note: For diluted samples, multiply the calculated concentration by the dilution factor.

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

Add 50 µL Detection Antibodies per well

Incubate 1 hour at RT

Do Not Aspirate

Add 50 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

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

Add 100 µL Sheath Fluid or Drive Fluid per well

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

PLATE WASHING

1.) Solid Plate

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

A.) Handheld magnet **(EMD Millipore Catalog #40-285)** - Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

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

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

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 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

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

These specifications are for the Luminex 200™, Luminex HTS, Luminex FLEXMAP 3D®, and Luminex MAGPIX® with xPONENT® software. Luminex instruments with other software (e.g. MasterPlex®, STarStation, LiquiChip, Bio-Plex Manager™, LABScan™ 100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex magnetic beads.

For magnetic bead assays, the Luminex 200™ and HTS instruments must be calibrated with the xPONENT® 3.1 compatible Calibration Kit (EMD Millipore Catalog #40-275) and performance verified with the Performance Verification Kit (EMD Millipore Catalog #40-276). The Luminex FLEXMAP 3D® instrument must be calibrated with the FLEXMAP 3D® Calibrator Kit (EMD Millipore Catalog #40-028) and performance verified with the FLEXMAP 3D® Performance Verification Kit (EMD Millipore Catalog #40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore Catalog #40-049) and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore Catalog #40-050).

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

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

EQUIPMENT SETTINGS (continued)

The Luminex probe height must be adjusted to the plate provided in the kit. Please use Cat.# MAG-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 9-plex Beads	
	NGF	20
	IL-6	34
	Insulin	36
	Leptin	39
	IL-8	44
	HGF	45
	MCP-1	52
	TNFα	55
	IL-1β	72

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.emdmillipore.com using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Cross-Reactivity

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

Assay Sensitivities (minimum detectable concentrations, pg/mL)

Minimum Detectable Concentration (MinDC) is calculated using MILLIPLEX® Analyst 5.1. 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 (n=8).

Analyte	MinDC (pg/mL)	MinDC+2SD (pg/mL)
NGF	0.3	0.7
IL-6	0.2	0.5
Insulin	3.8	13
Leptin	19	37
IL-8	0.3	0.5
HGF	4.0	9.8
MCP-1	1.2	2.2
TNFα	0.3	0.5
IL-1β	0.4	1.0

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	Intra-assay %CV	Inter-assay %CV
NGF	4	11
IL-6	2	10
Insulin	3	11
Leptin	5	13
IL-8	3	14
HGF	3	11
MCP-1	2	11
TNFα	3	19
IL-1β	7	12

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=5).

Analyte	% Recovery in serum matrix
NGF	107
IL-6	87
Insulin	94
Leptin	96
IL-8	87
HGF	94
MCP-1	101
TNFα	91
IL-1β	97

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 to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height to the kit solid plate using 1 alignment disc. For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.
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.

	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		
Problem	**Probable Cause**	**Solution**
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.
	Samples have insoluble particles	Centrifuge samples just prior to assay setup and use supernatant.
	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 #
Human Adipokine Panel 2 Standard	HADK2-8061-2
Human Adipokine Panel 2 Quality Controls 1 and 2	HADK2-6061-2
Human Adipokine Panel 2 Detection Antibodies	HADK2-1061-2
Serum Matrix	LHED-SD
Assay Buffer	LE-ABGLP
Bead Diluent	LHE-BD
Streptavidin-Phycoerythrin	L-SAPE7
Set of two 96-Well plates with sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Magnetic Beads

Analyte	Bead #	Cat. #
NGF	20	HNGF-MAG
IL-6	34	HIL6-MAG
Insulin	36	HINS-MAG
Leptin	39	HLPTN-MAG
IL-8	44	HIL8-MAG
HGF	45	HHGF-MAG
MCP-1	52	HMCP1-MAG
TNFα	55	HTNFA-MAG
IL-1β	72	HIL1B-MAG

ORDERING INFORMATION

To place an order:

To assure the clarity of your custom kit order, please FAX the following information to our customer service department:

Include:

- Your name, telephone and/or fax number
- Customer account number
- Shipping and billing address
- Purchase order number
- Catalog number and description of product
- Quantity of kits
- Selection of MILLIPLEX® Analytes

FAX: (636) 441-8050

Toll-Free US: (866) 441-8400
(636) 441-8400

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

For European Customers:

To best serve our European customers in placing an order or obtaining additional information about MILLIPLEX® MAP products, please contact your multiplex specialist or sales representative or email our European Customer Service at:

Austria	AUCustomerService@merckgroup.com
Belgium	BENLCustomerService@merckgroup.com
Denmark	denmark@merckgroup.com
France	FRCustomerService@merckgroup.com
Finland	Asiakaspalvelu@merckgroup.com
Germany	GECustomerService@merckgroup.com
Ireland	IECustomerService@merckgroup.com
Italy	CSR-IT@merckgroup.com
Netherlands	BENLCustomerService@merckgroup.com
Norway	Norway@merckgroup.com
Spain	pedidos@merckgroup.com
Sweden	Kundservice@merckgroup.com
Switzerland	SZCustomerService@merckgroup.com
UK	UKCustomerService@merckgroup.com

ORDERING INFORMATION (continued)

Conditions of Sale

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

Material Safety Data Sheets (MSDS)

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

Technical Services

http://www.emdmillipore.com/techservices

To contact by phone

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

Outside North America, contact your local office
http://www.emdmillipore.com/offices

WELL MAP

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

Human Circulating Cancer Biomarker Magnetic Bead Panel 1

96 Well Plate Assay

Cat. # HCCBP1MAG-58K

MILLIPLEX® MAP

HUMAN CIRCULATING CANCER BIOMARKER MAGNETIC BEAD PANEL 1

96 Well Plate Assay

HCCBP1MAG-58K

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

Human Circulating Cancer Biomarker Magnetic Bead Panel 1

INTRODUCTION

Cancer research has always focused on intracellular biomarkers, such as HER2 and B-Raf. However, in recent years more attention has shifted to include soluble cancer biomarkers. While the use of intracellular markers in research help clarify the process of oncogenesis, circulating cancer biomarkers give insight into how the body responds to the presence of a tumor, the dysregulation of homeostasis and the relationship between a tumor and its environment. Study of isolated biomarkers, whether intracellular or circulating, is often inadequate to analyze the complex relationship between tumor and non-tumor. Consequently, a large panel of cancer biomarkers better enables researchers to tease out these relationships and apply what they learn to understanding tumor biology.

MILLIPLEX® MAP offers the broadest selection of analytes across a wide range of disease states and species. Once the analytes of interest have been identified, you can rely on the quality that we build into each kit to produce results you can trust. In addition to the assay characteristics listed in the protocol, other performance criteria evaluated during the validation process include: cross-reactivity, dilution linearity, kit stability, and sample behavior (e.g. detectability and stability).

Each MILLIPLEX® MAP panel and kit includes:

- Quality controls (QCs) provided to qualify assay performance
- Comparison of standard (calibrator) and QC lots to a reference lot to ensure lot-to-lot consistency
- Optimized serum matrix to mimic native analyte environment
- Detection antibody cocktails designed to yield consistent analyte profiles within panel

In addition each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The MILLIPLEX® MAP Human Circulating Cancer Biomarker Magnetic Bead Panel thus enables you to focus on the therapeutic potential of cancer biomarkers. 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® MAP Circulating Cancer Biomarker Magnetic Bead Panel 1 is part of the most versatile system available for cancer research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically validate and build the most comprehensive library available for protein detection and quantitation.

- MILLIPLEX® MAP offers you:
    - The ability to choose any combination of analytes from our panel of 26 analytes to design a custom kit that better meets your needs.
    - A convenient "all-in-one" box format that gives you the assurance that you will have all the necessary reagents you need to run your assay.

EMD Millipore's MILLIPLEX® MAP Circulating Cancer Biomarker Magnetic Bead Panel 1 is a 26-plex kit that can be used for the simultaneous quantification of any or all of the following analytices: AFP, CA125, CA15-3, CA19-9, CEA, CYFRA21-1, sFas, sFasL, FGF2, β-HCG, HE4, HGF, IL-6, IL-8, Leptin, MIF, Osteopontin, Prolactin, PSA (free), PSA (total), SCF, TGFα, TNFα, TRAIL and VEGF.

Some biomarkers are tumor specific, such as PSA, while others, such as IL-8, have been detected in many cancers. Applicable sample types include serum, plasma and tissue/cell lysate and culture supernatant samples with the following exceptions:

- **PSA (free) and PSA (total) cannot be plexed together.**

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

- Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- EMD Millipore provides three Luminex instruments to acquire and analyze data using two detection methods:
    - The Luminex analyzers Luminex 200™ and FLEXMAP 3D®, flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
    - The Luminex analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. EMD Millipore combines the streamlined data acquisition power of Luminex xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex instruments.

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

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 – 8°C.
- Once the standards and controls have been reconstituted, immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSTITUTED 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 Antibodies, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
Human Circulating Cancer Biomarker Panel 1 Standard	HCC-8058	lyophilized	1 vial
Human Circulating Cancer Biomarker Panel 1 Quality Controls 1 and 2	HCC-6058	lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	HCP-SM	lyophilized	1 vial
Set of one 96-Well Plate with 2 Sealers	----------	----------	1 plate 2 sealers
Assay Buffer Note: Contains 0.08% Sodium Azide	L-AB	30 mL	2 bottles
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	2 bottles
Human Circulating Cancer Biomarker Panel 1 Detection Antibodies	HCC-1058	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE 4	3.2 mL	1 bottle
Bead Diluent	LBD-4	3.5 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

REAGENTS SUPPLIED (continued)

Human Circulating Cancer Biomarker Panel 1 Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable Beads (20X Concentration, 200 µL)	
		Available	Cat. #
Anti – AFP Bead	12	✓	HC1AFP-MAG
Anti – Total PSA Bead	13	✓	HTPSA-MAG
Anti – CA 15-3 Bead	15	✓	HCA153-MAG
Anti – CA 19-9 Bead	18	✓	HCA199-MAG
Anti – MIF Bead	20	✓	HMIF-MAG
Anti – TRAIL Bead	27	✓	HTRAIL-MAG
Anti – Leptin Bead	28	✓	HCCLPTN-MAG
Anti – Free PSA Bead	29	✓	HFPSA-MAG
Anti – IL-6 Bead	34	✓	HIL6-MAG
Anti – sFasL Bead	37	✓	HSFASLG-MAG
Anti – CEA Bead	39	✓	HCEA-MAG
Anti – CA125 Bead	42	✓	HCA125-MAG
Anti – IL-8 Bead	44	✓	HIL8-MAG
Anti – HGF Bead	45	✓	HHGF-MAG
Anti – sFas Bead	52	✓	HSFAS-MAG
Anti – TNFα Bead	55	✓	HTNFA-MAG
Anti – Prolactin Bead	56	✓	HCCPRLCTN-MAG
Anti – SCF Bead	61	✓	HSCF-MAG
Anti – CYFRA 21-1 Bead	63	✓	HCYFRA-MAG
Anti – OPN Bead	64	✓	H0PN-MAG
Anti – FGF2 Bead	67	✓	HFGF2-MAG
Anti – β HCG Bead	73	✓	BHCG-MAG
Anti – HE4 Bead	75	✓	HHE4-MAG
Anti – TGF α Bead	76	✓	HTGFA-MAG
Anti – VEGF Bead	78	✓	HVEGF-MAG

Note- 1. Total PSA and Free PSA cannot be run together in the same assay.

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (EMD Millipore Catalog #SHEATHFLUID) or Luminex Drive Fluid (EMD Millipore 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 (BioTek® 405 LS and 405 TS, EMD Millipore Catalog #40-094, #40-095, #40-096, #40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog #40-285 or equivalent).

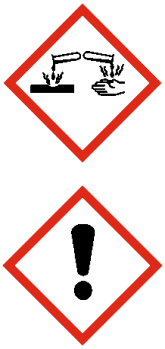
Note: If a plate washer or handheld magnetic separation block for magnetic beads is not available, one can use a microtiter filter plate (EMD Millipore Catalog #MX-PLATE) to run the assay using a Vacuum Filtration Unit (EMD Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with EMD Millipore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

SAFETY PRECAUTIONS

- All 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 buildup.

Note: See Full Labels of Hazardous components on next page.

Full Hazardous Components Labels:

Ingredient, Cat #		Full Label	
Human Circulating Cancer Biomarker Panel 1 Detection Antibodies	HCC-1058		**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.
Human Circulating Cancer Biomarker Panel 1 Quality Controls 1 & 2	HCC-6058		**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 Circulating Cancer Biomarker Panel 1 Standard	HCC-8058		**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	HCP-SM		Harmful to aquatic life with long lasting effects. Avoid release to the environment.
Bead Diluent	LBD-4		**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	L-SAPE4		**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.

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 an 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 ("Standard 7") which may be stored at ≤ -20°C for 1 month and at ≤ -80°C for greater than one month.
- If sample values 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 Mixing Bottle at 2-8°C for up to one month.
- During the preparation of the working standards, be certain to mix the higher concentration well before making the next dilution. In addition, use a new tip for each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some analytes.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.
- When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc.

For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.

- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells.
- 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 30 minutes at room temperature then centrifuge the samples for 10 minutes at 1000 x g. Finally, collect the serum samples and use them immediately in the assay or aliquot and store them 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 1:6 using the Serum Matrix provided in the kit as serum diluent. For a 1:6 dilution, add 15 µL of sample to 75 µL of Serum Matrix.
- For serum samples that require further dilution beyond 1:6, use the Serum Matrix provided in the kit for further dilution. Note: Matrix volume provided in the kit may be insufficient. Additional matrix may be obtained from EMD Millipore.
- For data analysis, multiply the final concentration of each sample by the dilution factor.

B. Preparation of Plasma Samples:

- Plasma collection using EDTA as an anticoagulant 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 1:6 using the Serum Matrix provided in the kit as sample diluent. For 1:6 dilution, add 15 µL of sample to 75 µL of Serum Matrix.

SAMPLE COLLECTION AND STORAGE (continued)

- For plasma samples that require further dilution beyond 1:6, use the Serum Matrix provided in the kit for further dilution. Note: Matrix volume provided in the kit may be insufficient. Additional matrix may be obtained from EMD Millipore.
- For data analysis, multiply the final concentration of each sample by the dilution factor.

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.

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 µL of blood collected.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

Sonicate each individual antibody-bead vial for 30 seconds then vortex for 1 minute. Add 150 µL from each antibody-bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent. 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 9 antibody-immobilized beads, add 150 µL from each of the 9 bead vials to the Mixing Bottle. Then add 1.65 mL Bead Diluent.

Example 2: When using 15 antibody-immobilized beads, add 150 µL from each of the 15 bead vials to the Mixing Bottle. Then add 0.75 mL Bead Diluent.

Example 3: When using ≥ 20 antibody-immobilized beads, add 150 µL from each of the bead vials to the Mixing Bottle. No additional Bead Diluent should be added.

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

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 then vortex briefly. Allow the vial to sit for 5-10 minutes. Unused portions 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 portions 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 1.0 mL deionized water to vial containing lyophilized Serum Matrix. Mix well. Allow at least 10 minutes for complete reconstitution. Then add 5.0 mL of assay buffer in the same vial to get a final volume of 6 mL of Serum Matrix. Left-over reconstituted Serum Matrix can be stored at ≤ -20°C for up to one month.

E. Preparation of Human Circulating Cancer Biomarker Panel 1 Standard

1.) Prior to use, reconstitute the Human Circulating Cancer Biomarker Panel 1 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. This will be used as Standard 7; the unused portion may be stored at ≤ -20°C for up to one month.

2.) Preparation of Working Standards

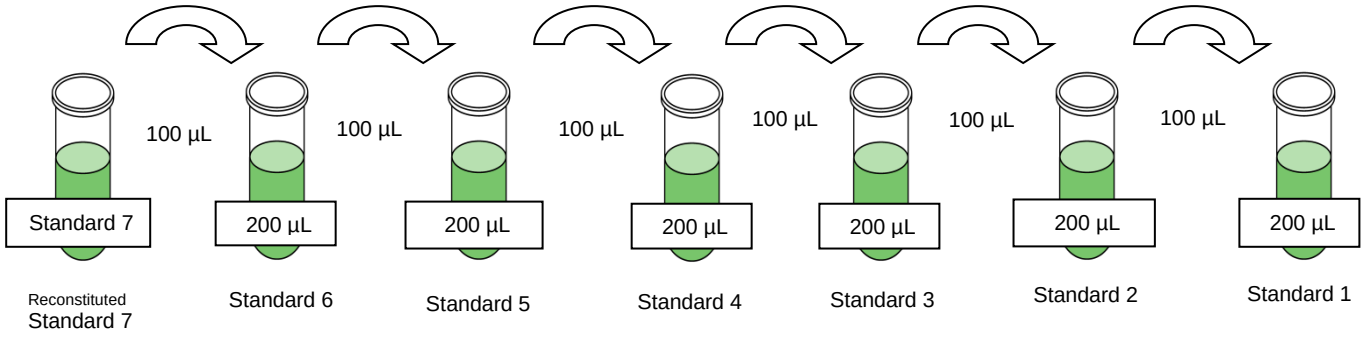
Label six polypropylene microfuge tubes as Standard 1, Standard 2, Standard 3, Standard 4, Standard 5, and Standard 6. Add 200 µL of Assay Buffer to each of the six tubes. Prepare 1:3 serial dilutions by adding 100 µL of the reconstituted Standard 7 to the Standard 6 tube, mix well and transfer 100 µL of the Standard 6 to the Standard 5 tube, mix well and transfer 100 µL of the Standard 5 to the Standard 4 tube, mix well and transfer 100 µL of the Standard 4 to Standard 3 tube, mix well and transfer 100 µL of the Standard 3 to the Standard 2 tube, mix well and transfer 100 µL of the Standard 2 to the Standard 1 tube and mix well. The 0 pg/mL Standard (Background) will be the Assay Buffer.

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

Standard (Tube #)	Volume of Deionized Water to Add	Volume of Standard to Add
Standard 7 (reconstituted standard)	250 µL	0

Standard (Tube #)	Volume of Assay Buffer to Add	Volume of Standard to Add
Standard 6	200 µL	100 µL of Standard 7
Standard 5	200 µL	100 µL of Standard 6
Standard 4	200 µL	100 µL of Standard 5
Standard 3	200 µL	100 µL of Standard 4
Standard 2	200 µL	100 µL of Standard 3
Standard 1	200 µL	100 µL of Standard 2

Preparation of Standards



After dilution, each tube has the following concentrations for each analyte:

Tube Number	Standard dilution	HE4 (pg/mL)	OPN (pg/mL)	AFP, Leptin, Prolactin (pg/mL)	sFas (pg/mL)	MIF, HGF (pg/mL)	Total PSA, sFasL, FGF-2, VEGF (pg/mL)
1	1:729	685.8	548.6	137.1	34.3	27.4	13.7
2	1:243	2057.8	1646.1	411.5	102.9	82.3	41.1
3	1:81	6172.8	4938.2	1234.5	308.7	246.9	123.4
4	1:27	18518.5	14814.8	3703.7	925.9	740.7	370.3
5	1:9	55555.5	44444.4	11111.1	2777.8	2222.2	1111.1
6	1:3	166666.6	133333.3	33333.3	8333.3	6666.6	3333.3
7	Original	500000	400000	100000	25000	20000	10000

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

Tube Number	Standard dilution	Free PSA, CA15-3, CA 19-9, CEA, CA125, CYFRA21-1	SCF (pg/mL)	TRAIL, TGF-α (pg/mL)	IL-8, TNFα (pg/mL)	IL-6 (pg/mL)	β-HCG (mU/mL)
1	1:729	Refer to QC analysis sheet for exact concentration	6.9	2.7	1.3	0.68	0.09
2	1:243		20.6	8.2	4.1	2.05	0.27
3	1:81		61.8	24.6	12.3	6.2	0.8
4	1:27		185.2	74.1	37	18.5	2.5
5	1:9		555.5	222.2	111.1	55.5	7.4
6	1:3		1666.6	666.6	333.3	166.6	22.3
7	Original		5000	2000	1000	500	67

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 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 samples in duplicate.
- If using a filter plate, set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Add 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of Assay Buffer to Background wells.
4. Add 25 µL of each Standard or Control into the appropriate wells.
5. Add 25 µL of appropriate matrix to Background, Standard 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 Assay Buffer to sample wells.
7. Add 25 µL of 1:6 diluted Sample into the appropriate wells. When assaying serum or plasma, use the Serum Matrix provided in the kit to dilute the sample. When assaying tissue culture or other supernatant, use proper control culture medium as the diluent.
8. Vortex Mixing Bottle and add 25 µL of the Mixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)

Add 200 µL Assay Buffer per well

Shake 10 min, RT

Decant

- Add 25 µL Assay Buffer to Background wells
- Add 25 µL Standard or Control to appropriate wells
- Add 25 µL appropriate matrix to Background, Standard and Control wells
- Add 25 µL Assay Buffer to sample wells
- Add 25 µL 1:6 diluted Sample to sample wells
- Add 25 µL Beads to each well

9. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-18 hr) at 4°C
10. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.
11. Add 25 µL of Detection Antibodies into each well.
12. Seal, cover with foil, and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT ASPIRATE AFTER INCUBATION.**
13. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.
14. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
15. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.
16. Add 100 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 5 minutes.
17. Run plate on Luminex 200™, HTS, FLEXMAP 3D® or MAGPIX® with xPONENT® software Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. (Note: Multiply the calculated concentration of the samples by the dilution factor, which is 6.

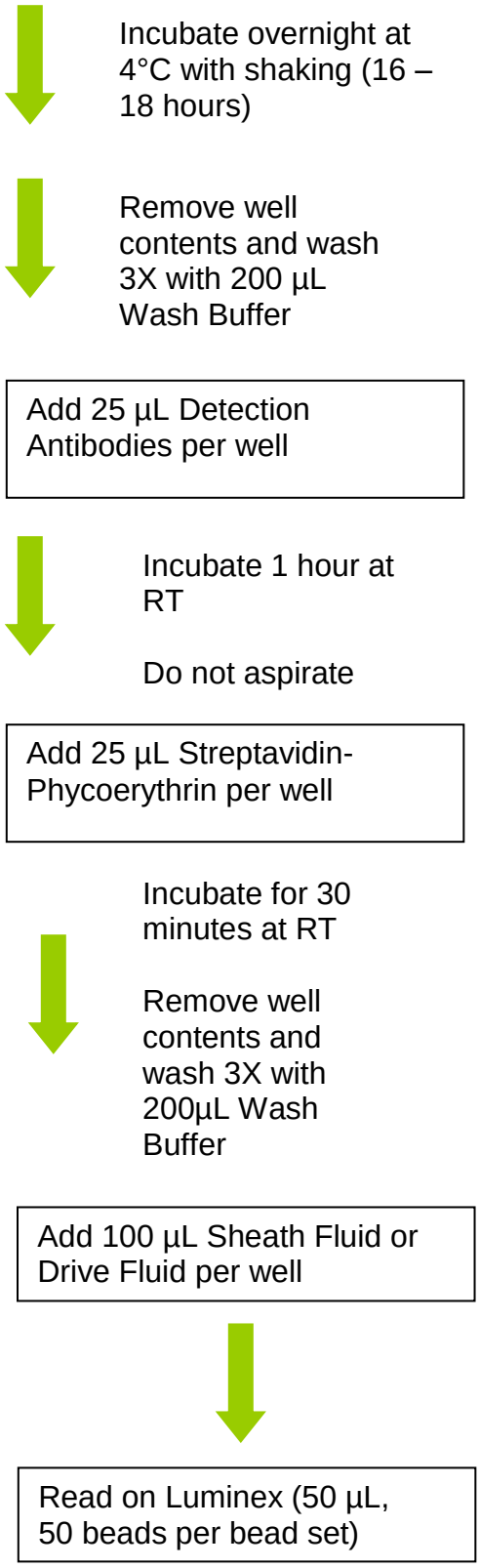


PLATE WASHING

1.) Solid Plate

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

A.) Handheld magnet **(EMD Millipore Catalog #40-285)** - Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

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

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

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 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

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

These specifications are for the Luminex 200™, Luminex HTS, Luminex FLEXMAP 3D®, and Luminex MAGPIX® with xPONENT® software. Luminex instruments with other software (e.g. MasterPlex®, STarStation, LiquiChip, Bio-Plex Manager™, LABScan™ 100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex magnetic beads.

For magnetic bead assays, the Luminex 200™ and HTS instruments must be calibrated with the xPONENT® 3.1 compatible Calibration Kit (EMD Millipore Catalog #40-275) and performance verified with the Performance Verification Kit (EMD Millipore Catalog #40-276). The Luminex FLEXMAP 3D® instrument must be calibrated with the FLEXMAP 3D® Calibrator Kit (EMD Millipore Catalog #40-028) and performance verified with the FLEXMAP 3D® Performance Verification Kit (EMD Millipore Catalog #40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore Catalog #40-049) and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore Catalog #40-050).

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

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

EQUIPMENT SETTINGS (continued)

The Luminex probe height must be adjusted to the plate provided in the kit. Please use Cat. # MAG-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:	Bead regions	
	AFP	12
	Total PSA	13
	CA15-3	15
	CA19-9	18
	MIF	20
	TRAIL	27
	Leptin	28
	Free PSA	29
	IL-6	34
	sFasL	37
	CEA	39
	CA125	42
	IL-8	44
	HGF	45
	sFas	52
	TNFα	55
	Prolactin	56
	SCF	61
	CYFRA 21-1	63
	OPN	64
	FGF2	67
	β-HCG	73
	HE4	75
	TGFα	76
	VEGF	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.emdmillipore.com using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Cross-Reactivity

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

Assay Sensitivities (minimum detectable concentrations, pg/mL)

Minimum Detectable Concentration (MinDC) is calculated using MILLIPLEX® Analyst 5.1. 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	*MinDC + 2SD (pg/mL)*	*MinDC + 2SD* (U/mL)	*MinDC + 2SD* (mU/mL)
AFP	74.7		
Total PSA	2.0		
MIF	7.6		
TRAIL	0.5		
Leptin	42.8		
Free PSA	1.4		
IL-6	0.2		
sFasL	3.7		
CEA	5.2		
IL-8	0.3		
HGF	6.8		
sFas	8.4		
TNFα	0.3		
Prolactin	30.2		
SCF	2.0		
CYFRA 21-1	59.3		
OPN	285.3		
FGF2	3.6		
HE4	193.5		
TGFα	0.5		
VEGF	6.4		
CA15-3		0.03	
CA19-9		0.3	
CA125		0.2	
β-HCG			0.029

N = 7 assays

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 assays. Inter-assay precision is generated from the mean of the %CV's across two different concentrations of analytes across six different assays.

Analyte	***Intra- assay %CV***	***Inter- assay %CV***
AFP	6	6.7
Total PSA	9	5.6
CA15-3	15	8.2
CA19-9	10.8	7.8
MIF	7.7	10.3
TRAIL	6.7	4.1
Leptin	4.9	6.7
Free PSA	8.9	7.6
IL-6	9.3	5.5
sFasL	7.0	6.4
CEA	11.3	4.6
CA125	6.5	5.3
IL-8	6.8	5.5
HGF	8.7	8.8
sFas	6.5	7.1
TNFα	7.9	7.5
Prolactin	7.6	5.5
SCF	10.4	7.5
CYFRA 21-1	6.7	16.2
OPN	8.9	6.0
FGF2	7.9	6.3
β-HCG	7.5	5.3
HE4	9.3	6.4
TGFα	8.7	4.9
VEGF	12	10.2

ASSAY CHARACTERISTICS (continued)

Accuracy

Spike Recovery: The data represent mean percent recovery of 3 levels of spiked standards in serum samples (n=5).

Analyte	Spike Recovery in Serum
AFP	86
Total PSA	59
CA15-3	77
CA19-9	58
MIF	50
TRAIL	91
Leptin	81
Free PSA	59
IL-6	89
sFasL	63
CEA	74
CA125	68
IL-8	51
HGF	77
sFas	69
TNFα	70
Prolactin	108
SCF	68
CYFRA 21-1	97
OPN	71
FGF2	85
β-HCG	69
HE4	77
TGFα	76
VEGF	74

ASSAY CHARACTERISTICS (continued)

Cell Culture Analysis

The following human cell lines were cultured according to the recommendations of the ATCC to around 80% confluence; then the respective media was centrifuged and run in the assay.

- A431 – Epidermoid Carcinoma
- Daudi – Burkitt's lymphoma
- HeLa – Cervical Adenocarcinoma
- HepG2 – Hepatocellular Carcinoma
- HuVec – Umbilical vein endothelial cell
- Jurkat – Lymphoblast
- LnCap – Prostate Adenocarcinoma
- PC-3 – Prostate Adenocarcinoma
- SW116 – Colon Carcinoma
- ZR75-1 – Mammary gland Carcinoma

	A431	Daudi	HeLa	HepG2	HuVec	Jurkat	LnCap	PC-3	SW116	ZR75-1
AFP				H						L
CA125	L	L	M						L	L
CA15-3										L
CA19-9	L	L		L					M	M
CEA	L							L	H	M
CYFRA21-1	L		L	H	L			M	H	H
FGF2					M					
β-HCG	M		L						L	L
HE4										L
HGF										
IL-6	L		M		H			M	L	L
IL-8	L		L	M	H		L	M	M	L
Leptin										L
MIF		H			H	M	M		M	H
OPN	L	L	L	H		L			L	L
Prolactin		L								L
Total PSA							H			L
sFas				L	L		L		L	L
sFasL									L	M
SCF										L
TGFα				L				L	L	L
TNFα		L							L	L
TRAIL	L				L					L
VEGF	M	L	L	H			H	L	L	M

Blank- Not detected

L – Values in the lower third of the respective analyte standard curve

M – Values in the mid third of the respective analyte standard curve

H – Values in the upper third of the respective analyte standard curve

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 to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height to the kit solid plate using 1 alignment disc. For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately, and pipetting 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°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.
	Beads were exposed to light	Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.

Problem	Probable Cause	Solution
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		
Problem	**Probable Cause**	**Solution**
Filter plate will not vacuum	Vacuum pressure is insufficient	Increase vacuum pressure such that 0.2 µL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble particles	Centrifuge samples just prior to assay setup and use supernatant.
	High lipid concentration	After centrifugation, remove lipid layer and use supernatant.
Plate leaked	Vacuum Pressure too high	Adjust vacuum pressure such that 0.2 µL 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

Components	Catalog #
Human Circulating Cancer Biomarker Panel 1 Standard	HCC-8058
Human Circulating Cancer Biomarker Panel 1 Quality Controls 1 and 2	HCC-6058
Serum Matrix	HCP-SM
Human Circulating Cancer Biomarker Panel 1 Detection Antibodies	HCC-1058
Streptavidin-Phycoerythrin	L-SAPE 4
Assay Buffer	L-AB
Bead Diluent	LBD-4
96-Well Plate with two Sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Magnetic Beads

Analyte	*Bead #*	*Catalog #*
AFP	12	HC1AFP-MAG
Total PSA	13	HTPSA-MAG
CA15-3	15	HCA153-MAG
CA19-9	18	HCA199-MAG
MIF	20	HMIF-MAG
TRAIL	27	HTRAIL-MAG
Leptin	28	HCCLPTN-MAG
Free PSA	29	HFPSA-MAG
IL-6	34	HIL6-MAG
sFasL	37	HSFASLG-MAG
CEA	39	HCEA-MAG
CA125	42	HCA125-MAG
IL-8	44	HIL8-MAG
HGF	45	HHGF-MAG
sFas	52	HSFAS-MAG
TNFα	55	HTNFA-MAG
Prolactin	56	HCCPRLCTN-MAG
SCF	61	HSCF-MAG
CYFRA 21-1	63	HCYFRA-MAG
OPN	64	H0PN-MAG
FGF2	67	HFGF2-MAG
β-HCG	73	BHCG-MAG
HE4	75	HHE4-MAG
TGFα	76	HTGFA-MAG
VEGF	78	HVEGF-MAG

ORDERING INFORMATION

To place an order:

To assure the clarity of your custom kit order, please FAX the following information to our customer service department:

Include:

- Your name, telephone and/or fax number
- Customer account number
- Shipping and billing address
- Purchase order number
- Catalog number and description of product
- Quantity of kits
- Selection of MILLIPLEX® Analytes

FAX: (636) 441-8050

Toll-Free US: (866) 441-8400
(636) 441-8400

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

For European Customers:

To best serve our European customers in placing an order or obtaining additional information about MILLIPLEX® MAP products, please contact your multiplex specialist or sales representative or email our European Customer Service at:

Austria	AUCustomerService@merckgroup.com
Belgium	BENLCustomerService@merckgroup.com
Denmark	denmark@merckgroup.com
France	FRCustomerService@merckgroup.com
Finland	Asiakaspalvelu@merckgroup.com
Germany	GECustomerService@merckgroup.com
Ireland	IECustomerService@merckgroup.com
Italy	CSR-IT@merckgroup.com
Netherlands	BENLCustomerService@merckgroup.com
Norway	Norway@merckgroup.com
Spain	pedidos@merckgroup.com
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Switzerland	SZCustomerService@merckgroup.com
UK	UKCustomerService@merckgroup.com

ORDERING INFORMATION (continued)

Conditions of Sale

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Material Safety Data Sheets (MSDS)

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

Technical Services

http://www.emdmillipore.com/techservices

To contact by phone

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

Outside North America, contact your local office
http://www.emdmillipore.com/offices

WELL MAP

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

Human Cytokine/Chemokine Magnetic Bead Panel

96 Well Plate Assay

Cat. # HCYTOMAG-60K
HCYTMAG-60K-PX29
HCYTMAG-60K-PX30
HCYTMAG-60K-PX38
HCYTMAG-60K-PX41

MILLIPLEX® MAP

HUMAN CYTOKINE / CHEMOKINE MAGNETIC BEAD PANEL
96 Well Plate Assay

HCYTOMAG-60K or
HCYTMAG-60K-PX29
HCYTMAG-60K-PX30
HCYTMAG-60K-PX38
HCYTMAG-60K-PX41

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Human Cytokine / Chemokine Magnetic Bead Panel Kit

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.

MILLIPLEX® MAP offers the broadest selection of analytes across a wide range of disease states and species. Once the analytes of interest have been identified, you can rely on the quality that we build into each kit to produce results you can trust. In addition to the assay characteristics listed in the protocol, other performance criteria evaluated during the validation process include: cross-reactivity, dilution linearity, kit stability, and sample behavior (e.g. detectability and stability).

Each MILLIPLEX® MAP panel and kit includes:

- Quality controls (QCs) provided to qualify assay performance
- Comparison of standard (calibrator) and QC lots to a reference lot to ensure lot-to-lot consistency
- Optimized serum matrix to mimic native analyte environment
- Detection antibody cocktails designed to yield consistent analyte profiles within panel

In addition each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The **MILLIPLEX® MAP** Human Cytokine / Chemokine Magnetic Bead Panel thus 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 dozens of analytes simultaneously, which can dramatically improve productivity.

EMD Millipore's MILLIPLEX® MAP Human Cytokine / Chemokine Magnetic Bead Panel is part of the most versatile system available for cytokine and chemokine research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically validate and build the most comprehensive library available for protein detection and quantitation.

- MILLIPLEX® MAP offers you:
    - The ability to select a 38-plex (for serum/plasma) or 41-plex (for cell culture) pre-mixed kit
    - The ability to choose any combination of analytes from our panel of 41 analytes to design a custom kit that better meets your needs.
    - A convenient "all-in-one" box format that gives you the assurance that you will have all the necessary reagents you need to run your assay.

EMD Millipore's MILLIPLEX® MAP Human Cytokine / Chemokine Panel is to be used for the simultaneous quantification of the following 41 human cytokines and chemokines in human tissue/cell lysate and culture supernatant samples and serum or plasma samples: EGF, Eotaxin, G-CSF, GM-CSF, IFNα2, IFNγ, IL-10, IL-12P40, IL-12P70, IL-13, IL-15, IL-17A, IL-1RA, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, TNFα, TNFβ, VEGF,FGF-2, TGF-α, FIT-3L, Fractalkine, GRO, MCP-3, MDC, PDGF-AA, PDGF-AB/BB, sCD40L, and IL-9.

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

Please read entire protocol before use.

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

PRINCIPLE

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

- Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- EMD Millipore provides three Luminex instruments to acquire and analyze data using two detection methods:
    - The Luminex analyzers Luminex 200™ and FLEXMAP 3D®, flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
    - The Luminex analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. EMD Millipore combines the streamlined data acquisition power of Luminex xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex instruments.

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

STORAGE CONDITIONS UPON RECEIPT

Recommended storage for kit components is 2 - 8°C.

- 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
Human Cytokine / Chemokine Standard	MXH8060-2 (for 29, 30plex) or MXH8060 (for 38, 41plex)	lyophilized	1 vial
Human Cytokine Quality Controls 1 and 2	MXH6060-2 (for 29, 30plex) or MXH6060 (for 38, 41plex)	lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	MXHSM	lyophilized	1 vial (required for serum and plasma samples only)
Set of one 96-Well Plates with 2 Sealers	----------	----------	1 plates 2 sealers
Assay Buffer	L-AB	30 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	60 mL	1 bottle
Human Cytokine Detection Antibodies	MXH1060-1 or MXH1060-2 or MXH1060-3 or MXH1060-4	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE9 *(Use with Cat. # MXH1060-1)* or L-SAPE3 *(Use with Cat. # MXH1060-2)* or L-SAPE10 *(Use with Cat. # MXH1060-3)* or L-SAPE11 *(Use with Cat. # MXH1060-4)*	3.2 mL	1 bottle
Bead Diluent (not provided with premixed panel)	LBD	3.5 mL	1 bottle
Mixing Bottle (not provided with premixed panel)	----------	----------	1 bottle

REAGENTS SUPPLIED (continued)

Human Cytokine / Chemokine Antibody-Immobilized Premixed Magnetic Beads:

Premixed 29-plex Beads	HCYPMX29-MAG	3.5 mL	1 bottle
Premixed 30-plex Beads (Premixed 29plex + RANTES)	HCYPMX29-MAG+HCYRNTS-MAG	3.5 mL	1 bottle + 1 vial
Premixed 38-plex Beads	HCYPMX38-MAG	3.5 mL	1 bottle
Premixed 41-plex Beads (premixed 38-plex + RANTES, PDGF-AA, PDGF-AB/BB)	HCYPMX38-MAG + HCYRNTS-MAG, HPDGFAA-MAG, HPDGFBB-MAG	3.5 mL	1 bottle + 3 vials

Included Human Cytokine / Chemokine Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel (see below).

Human Cytokine / Chemokine Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable 41 Analytes (50X concentration, 90µL) Available	Cat. #	29-Plex Premixed Beads	38-Plex Magnetic Premixed Beads	41-Plex Premixed Beads
Anti-Human EGF Bead	12	✓	HEGF-MAG	✓	✓	✓
Anti-Human FGF-2 Bead	13	✓	HCYFGF2-MAG		✓	✓
Anti-Human Eotaxin Bead	14	✓	HETXN-MAG	✓	✓	✓
Anti-Human TGF-α Bead	15	✓	HCYTGFA-MAG		✓	✓
Anti-Human G-CSF Bead	18	✓	HGCSF-MAG	✓	✓	✓
Anti-Human Flt-3L Bead	19	✓	HFLT3L-MAG		✓	✓
Anti-Human GM-CSF Bead	20	✓	HGMCSF-MAG	✓	✓	✓
Anti-Human Fractalkine Bead	21	✓	HFKN-MAG		✓	✓
Anti-Human IFNα2 Bead	22	✓	HIFNA2-MAG	✓	✓	✓
Anti-Human IFNγ Bead	25	✓	HCYIFNG-MAG	✓	✓	✓
Anti-Human GRO Bead	26	✓	HGR0-MAG		✓	✓
Anti-Human IL-10 Bead	27	✓	HCYIL10-MAG	✓	✓	✓
Anti-Human MCP-3 Bead	28	✓	HMCP3-MAG		✓	✓
Anti-Human IL-12p40 Bead	29	✓	HIL12P40-MAG	✓	✓	✓

Human Cytokine / Chemokine Antibody-Immobilized Magnetic Beads (continued):

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable 41 Analytes (50X concentration, 90µL) Available	Cat. #	29-Plex Premixed Beads	38-Plex Magnetic Premixed Beads	41-Plex Premixed Beads
Anti-Human MDC Bead	30	✓	HMDC-MAG		✓	✓
Anti-Human IL-12P70 Bead	33	✓	HIL12P70-MAG	✓	✓	✓
Anti-Human PDGF-AA Bead	34	✓	HPDGFAA-MAG			✓
Anti-Human IL-13 Bead	35	✓	HIL13-MAG	✓	✓	✓
Anti-Human PDGF-AB/BB Bead	36	✓	HPDGFBB-MAG			✓
Anti-Human IL-15 Bead	37	✓	HIL15-MAG	✓	✓	✓
Anti-Human sCD40L Bead	38	✓	HCD40L-MAG		✓	✓
Anti-Human IL-17A Bead	39	✓	HIL17-MAG	✓	✓	✓
Anti-Human IL-1RA Bead	42	✓	HIL1RA-MAG	✓	✓	✓
Anti-Human IL-1α Bead	44	✓	HIL1A-MAG	✓	✓	✓
Anti Human IL-9 Bead	45	✓	HIL9-MAG		✓	✓
Anti-Human IL-1β Bead	46	✓	HCYIL1B-MAG	✓	✓	✓
Anti-Human IL-2 Bead	48	✓	HIL2-MAG	✓	✓	✓
Anti-Human IL-3 Bead	51	✓	HIL3-MAG	✓	✓	✓
Anti-Human IL-4Bead	53	✓	HIL4-MAG	✓	✓	✓
Anti-Human IL-5 Bead	55	✓	HIL5-MAG	✓	✓	✓
Anti-Human IL-6 Bead	57	✓	HCYIL6-MAG	✓	✓	✓
Anti-Human IL-7 Bead	61	✓	HIL7-MAG	✓	✓	✓
Anti-Human IL-8 Bead	63	✓	HCYIL8-MAG	✓	✓	✓
Anti-Human IP-10 Bead	65	✓	HIP10-MAG	✓	✓	✓
Anti-Human MCP-1 Bead	67	✓	HCYMCP1-MAG	✓	✓	✓
Anti-Human MIP-1αBead	72	✓	HMIP1A-MAG	✓	✓	✓
Anti-Human MIP-1β Bead	73	✓	HMIP1B-MAG	✓	✓	✓
Anti-Human RANTES Bead	74	✓	HCYRNTS-MAG			✓
Anti-Human TNFα Bead	75	✓	HCYTNFA-MAG	✓	✓	✓
Anti-Human TNFβ Bead	76	✓	HTNFB-MAG	✓	✓	✓
Anti-Human VEGF Bead	78	✓	HCYVEGF-MAG	✓	✓	✓

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (EMD Millipore Catalog #SHEATHFLUID) or Luminex Drive Fluid (EMD Millipore 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 (BioTek® 405 LS and 405 TS, EMD Millipore Catalog #40-094, #40-095, #40-096, #40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog #40-285 or equivalent).

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

SAFETY PRECAUTIONS

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Note: See Full Labels of Hazardous components on next page.

Full Labels of Hazardous components:

Ingredient, Cat #		Full Label	
Streptavidin-Phycoerythrin	L-SAPE10		**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	L-SAPE11		**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	L-SAPE3		**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	L-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.
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.
Serum Matrix	MXHSM		Harmful to aquatic life with long lasting effects. Avoid release to the environment.
Human Cytokine Detection Antibodies	MXH1060-1		**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.
Human Cytokine Detection Antibodies	MXH1060-2		**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.
Human Cytokine Detection Antibodies	MXH1060-3		**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.

Full Labels of Hazardous components continued:

Ingredient, Cat #		Full Label	
Human Cytokine/Chemokine Quality Control 1 & 2	MXH6060		**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 Cytokine/Chemokine Quality Control 1 & 2	MXH6060-2		**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 Cytokine/Chemokine Standard	MXH8060		**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 Cytokine/Chemokine Standard	MXH8060-2		**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.

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.
- When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc.

  For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.

TECHNICAL GUIDELINES (continued)

- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.
- 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 serum/plasma sample that require a dilution instead of "Neat", use the Serum Matrix provided in the kit as the diluent.
- 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.
- Neat Serum samples (for measuring 38 cytokines, not including RANTES, PDGF-AA, PDGF-AB/BB) are used. When further dilution is required, use Serum Matrix as the diluent.
- When measuring RANTES, PDGF-AA, PDGF-AB/BB in serum, samples should be diluted 1:100 in the Assay Buffer and a standard curve with Assay **Buffer matrix should be used accordingly**. When further dilution beyond 1:100 is required, use Assay Buffer 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.
- Neat Plasma samples (for measuring 38 cytokines, not including RANTES, PDGF-AA, PDGF-AB/BB) are used. When further dilution is required, use Serum Matrix as the diluent.

SAMPLE COLLECTION AND STORAGE (continued)

- When measuring RANTES, PDGF-AA, PDGF-AB/BB in plasma, sample should be diluted 1:100 in the Assay Buffer and a standard curve with Assay B**uffer matrix should be used accordingly**. When further dilution beyond 1:100 is required, use Assay Buffer 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 neat or 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.
To prepare 41 plex premixed beads, add 70 µL of RANTES, PDGF-AA and PDGF-AB/BB beads to the 38-plex premixed bead bottle. Mix well before use.
(**Note**: Due to high concentration of RANTES, PDGF-AA, PDGF-AB/BB in serum/plasma, they have to be measured separately with **1:100** diluted serum/plasma. 38plex premixed beads are used for measuring all other 38 cytokines in serum/plasma with **Neat** serum/plasma)

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 Bead Diluent. 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 cytokine antibody-immobilized beads, add 60 µL from each of the 20 bead sets to the Mixing Bottle. Then add 1.8 mL Bead Diluent.

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 Bead Diluent.

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

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

E. Preparation of Human Cytokine Standard

1.) Prior to use, reconstitute the Human Cytokine Standard with 250 µL deionized water to give a 10,000 pg/mL concentration of standard for all 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 10,000 pg/mL standard; the unused portion may be stored at ≤ -20°C for up to one month.

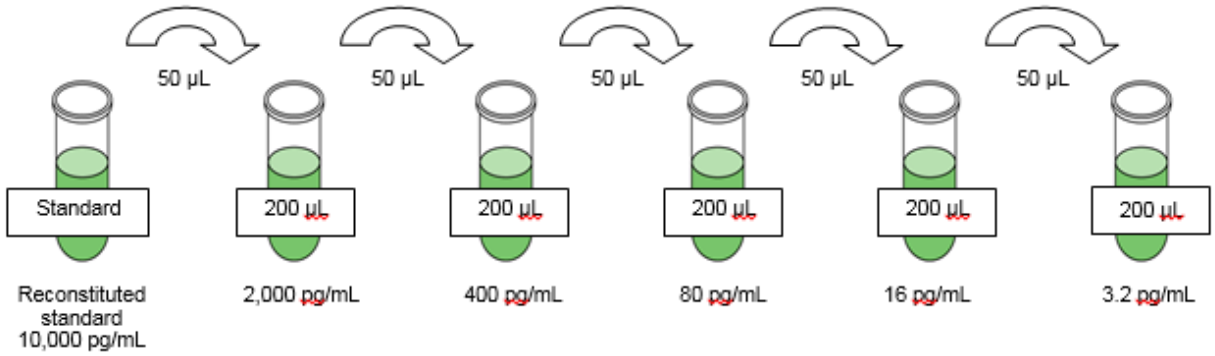
2). Preparation of Working Standards

Label five polypropylene microfuge tubes 2,000, 400, 80, 16, and 3.2 pg/mL. Add 200 µL of Assay Buffer to each of the five tubes. Prepare serial dilutions by adding 50 µL of the 10,000 pg/mL reconstituted standard to the 2,000 pg/mL tube, mix well and transfer 50 µL of the 2,000 pg/mL standard to the 400 pg/mL tube, mix well and transfer 50 µL of the 400 pg/mL standard to the 80 pg/mL tube, mix well and transfer 50 µL of the 80 pg/mL standard to 16 pg/mL tube, mix well and transfer 50 µL of the 16 pg/mL standard to the 3.2 pg/mL tube and mix well. The 0 pg/mL standard (Background) will be Assay Buffer.

Standard Concentration (pg/mL)	Volume of Deionized Water to Add	Volume of Standard to Add
10,000	250 µL	0

Standard Concentration (pg/mL)	Volume of Assay Buffer to Add	Volume of Standard to Add
2,000	200 µL	50 µL of 10,000 pg/mL
400	200 µL	50 µL of 2000 pg/mL
80	200 µL	50 µL of 400 pg/mL
16	200 µL	50 µL of 80 pg/mL
3.2	200 µL	50 µL of 16 pg/mL

Preparation of Standards



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), 3.2, 16, 80, 400, 2,000, and 10,000 pg/mL], 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 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 serum/plasma Sample (1:100 dilution for RANTES, PDGF-AA, and PDGF-AB/BB, Neat for all other 38 cytokines) or 25 µl cell culture 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.)
8. Seal the plate with a plate sealer. Wrap the plate with foil 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 Solution to background, standards, and control wells
- Add 25 µL Samples to sample wells
- Add 25 µL Beads to each well

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

9. Gently remove well contents and wash plate 2 times following instructions listed in the **PLATE WASHING** section**.**
10. Add 25 µL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)
11. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT ASPIRATE AFTER INCUBATION.**
12. Add 25 µL Streptavidin-Phycoerythrin to each well containing the 25 µL of Detection Antibodies.
13. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
14. Gently remove well contents and wash plate 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 on a plate shaker for 5 minutes.
16. Run plate on Luminex 200™, HTS, FLEXMAP 3D® or MAGPIX® with xPONENT® software.
17. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating cytokine/chemokines concentrations in samples. (Note: For diluted samples, multiply the calculated concentration by the dilution factor.)

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

Add 25 µL Detection Antibodies per well

Incubate 1 hour at RT

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 handheld magnet or magnetic plate washer.

A.) Handheld magnet **(EMD Millipore Catalog #40-285)** - Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

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

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

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 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

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

These specifications are for the Luminex 200™, Luminex HTS, Luminex FLEXMAP 3D®, and Luminex MAGPIX® with xPONENT® software. Luminex instruments with other software (e.g. MasterPlex®, STarStation, LiquiChip, Bio-Plex Manager™, LABScan™ 100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex magnetic beads.

For magnetic bead assays, the Luminex 200™ and HTS instruments must be calibrated with the xPONENT® 3.1 compatible Calibration Kit (EMD Millipore Catalog #40-275) and performance verified with the Performance Verification Kit (EMD Millipore Catalog #40-276). The Luminex FLEXMAP 3D® instrument must be calibrated with the FLEXMAP 3D® Calibrator Kit (EMD Millipore Catalog #40-028) and performance verified with the FLEXMAP 3D® Performance Verification Kit (EMD Millipore Catalog #40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore Catalog #40-049) and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore Catalog #40-050).

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

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

EQUIPMENT SETTINGS (continued)

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

Events:	50, per bead	
Sample Size:	100 µL	
Gate Settings:	8,000 to 15,000	
Reporter Gain:	Default (low PMT)	
Time Out:	60 seconds	
Bead Set:	Customizable 41-Plex Beads	
	EGF	12
	FGF-2	13
	Eotaxin	14
	TGF-α	15
	G-CSF	18
	Flt-3L	19
	GM-CSF	20
	Fractalkine	21
	IFNα2	22
	IFNγ	25
	GRO	26
	IL-10	27
	MCP-3	28
	IL-12P40	29
	MDC	30
	IL-12P70	33
	PDGF-AA	34
	IL-13	35
	PDGF-AB/BB	36
	IL-15	37
	sCD40L	38
	IL-17A	39
	IL-1RA	42
	IL-1α	44
	IL-9	45
	IL-1β	46
	IL-2	48
	IL-3	51
	IL-4	53
	IL-5	55
	IL-6	57
	IL-7	61
	IL-8	63
	IP-10	65
	MCP-1	67
	MIP-1α	72
	MIP-1β	73
	RANTES	74
	TNFα	75
	TNFβ	76
	VEGF	78

QUALITY CONTROLS

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located the EMD MILLIPORE website www.emdmillipore.com 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)

Minimum Detectable Concentration (MinDC) is calculated using MILLIPLEX® Analyst 5.1. 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.

ASSAY CHARACTERISTICS (continued)

Assay Sensitivities (minimum detectable concentrations, pg/mL)

Cytokine	MinDC (pg/ml)	MinDC+2SD (pg/ml)
EGF	2.8	4.6
FGF-2	7.6	11.8
Eotaxin	4.0	6.8
TGFα	0.8	1.2
G-CSF	1.8	3.3
Flt-3L	5.4	7.0
GM-CSF	7.5	15.0
Fractalkine	22.7	37.7
IFNα2	2.9	4.8
IFNγ	0.8	1.1
GRO	9.9	14.1
IL-10	1.1	1.6
MCP-3	3.8	6.4
IL-12P40	7.4	12.7
MDC	3.6	7.1
IL-12P70	0.6	1.0
IL-13	1.3	1.9
IL-15	1.2	1.7
sCD40L	5.1	9.9
IL-17	0.7	1.2
IL-1RA	8.3	17.1
IL-1α	9.4	12.6
IL-9	1.2	2.0
IL-1β	0.8	1.0
IL-2	1.0	1.6
IL-3	0.7	1.0
IL-4	4.5	7.1
IL-5	0.5	0.7
IL-6	0.9	1.3
IL-7	1.4	2.4
IL-8	0.4	0.7
IP-10	8.6	14.0
MCP-1	1.9	3.4
MIP-1α	2.9	6.2
MIP-1β	3.0	4.8
TNFα	0.7	1.1
TNFβ	1.5	1.9
VEGF	26.3	47.9
PDGF-AA	0.4	0.7
PDGFAB-BB	2.2	2.7
RANTES	1.2	1.9

ASSAY CHARACTERISTICS (continued)

Precision

Intra-assay precision is generated from the mean of the % CV's from sixteen 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 four reportable results across two different concentrations of cytokines across six different experiments.

Cytokine	Intra-assay %CV	Inter-assay %CV (N=6 assays)
EGF	2.3	5.8
FGF-2	2.3	4.8
Eotaxin	7.2	10.8
TGFα	4.1	9.5
G-CSF	1.8	15.5
Flt-3L	2.4	6.6
GM-CSF	3.1	10.1
Fractalkine	4.5	9.4
IFNα2	2.4	13.3
IFNγ	1.6	12.0
GRO	2.1	9.2
IL-10	1.6	16.8
MCP-3	1.6	6.4
IL-12P40	2.8	12.4
MDC	1.6	7.2
IL-12P70	2.2	16.7
IL-13	2.2	9.2
IL-15	2.7	8.1
sCD40L	3.7	18.9
IL-17	2.2	7.9
IL-1RA	2.1	10.7
IL-1α	3.3	12.8
IL-9	2.4	8.4
IL-1β	2.3	6.7
IL-2	2.1	6.3
IL-3	3.4	6.1
IL-4	2.9	14.2
IL-5	2.6	10.8
IL-6	2.0	18.3
IL-7	1.7	16.1
IL-8	1.9	3.5
IP-10	2.6	15.3
MCP-1	1.5	7.9
MIP-1α	1.9	14.5
MIP-1β	2.4	8.8
TNFα	2.6	13.0
TNFβ	1.6	11.4
VEGF	3.7	10.4
PDGF-AA	4.3	16.7
PDGFAB-BB	2.1	12.3
RANTES	1.9	5.0

ASSAY CHARACTERISTICS (continued)

Accuracy

Spike Recovery: The data represents mean recovery of three concentration levels (low, medium and high) of spiked standards ranging from 3-10,000pg/mL in serum matrix.

Cytokine	% Recovery in matrix
EGF	97.5
FGF-2	99.0
Eotaxin	100.5
TGFα	91.7
G-CSF	100.3
Flt-3L	98.2
GM-CSF	100.7
Fractalkine	87.2
IFNα2	93.9
IFNγ	98.1
GRO	97.5
IL-10	97.7
MCP-3	97.0
IL-12P40	93.3
MDC	102.3
IL-12P70	104.0
IL-13	95.0
IL-15	95.3
sCD40L	95.2
IL-17A	103.8
IL-1RA	93.5
IL-1α	92.9
IL-9	99.4
IL-1β	94.9
IL-2	95.4
IL-3	101.0
IL-4	94.5
IL-5	99.9
IL-6	96.1
IL-7	93.0
IL-8	98.3
IP-10	93.8
MCP-1	98.3
MIP-1α	105.0
MIP-1β	92.4
TNFα	97.8
TNFβ	97.5
VEGF	91.8
PDGF-AA	97.9
PDGFAB-BB	102.0
RANTES	93.8

ASSAY CHARACTERISTICS (continued)

Accuracy (continued)

Cytokine	% Recovery in matrix
EGF	97.5
FGF-2	99.0
Eotaxin	100.5
TGFα	91.7
G-CSF	100.3
Flt-3L	98.2
GM-CSF	100.7
Fractalkine	87.2
IFNα2	93.9
IFNγ	98.1
GRO	97.5
IL-10	97.7
MCP-3	97.0
IL-12P40	93.3
MDC	102.3
IL-12P70	104.0
IL-13	95.0
IL-15	95.3
sCD40L	95.2
IL-17A	103.8
IL-1RA	93.5
IL-1α	92.9
IL-9	99.4
IL-1β	94.9
IL-2	95.4
IL-3	101.0
IL-4	94.5
IL-5	99.9
IL-6	96.1
IL-7	93.0
IL-8	98.3
IP-10	93.8
MCP-1	98.3
MIP-1α	105.0
MIP-1β	92.4
TNFα	97.8
TNFβ	97.5
VEGF	91.8
PDGF-AA	97.9
PDGFAB-BB	102.0
RANTES	93.8

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 to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height to the kit solid plate using 1 alignment disc. For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.
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°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.
	Beads were exposed to light	Keep plate and bead mix covered with dark lid or aluminum foil during all incubation steps.

Problem	Probable Cause	Solution
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		
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 setup and use supernatant.
	High lipid concentration	After centrifugation, remove lipid layer and use supernatant.
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 (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	Cat #
Human Cytokine / Chemokine Standard	MXH8060
Human Cytokine / Chemokine Standard	MXH8060-2
Human Cytokine Quality Controls 1 and 2	MXH6060
Human Cytokine Quality Controls 1 and 2	MXH6060-2
Human Cytokine Detection Antibodies	MXH1060-1
Human Cytokine Detection Antibodies	MXH1060-2
Human Cytokine Detection Antibodies	MXH1060-3
Human Cytokine Detection Antibodies	MXH1060-4
Serum Matrix	MXHSM
Bead Diluent	LBD
Assay Buffer	L-AB
Streptavidin-Phycoerythrin	L-SAPE9
Streptavidin-Phycoerythrin	L-SAPE3
Streptavidin-Phycoerythrin	L-SAPE10
Streptavidin-Phycoerythrin	L-SAPE11
Set of two 96-Well Black plates with sealers	MAG-PLATE
10X Wash Buffer	L-WB
Human Cytokine/Chemokine 29 Plex Premixed Magnetic Bead Panel – BULK PACKAGING	HCYTMAG60PMX29BK
Human Cytokine/Chemokine 30 Plex Premixed Magnetic Bead Panel – BULK PACKAGING	HCYTMAG60PMX30BK
Human Cytokine/Chemokine 38 Plex Premixed Magnetic Bead Panel – BULK PACKAGED	HCYTMAG60PMX38BK
Human Cytokine/Chemokine 41 Plex Premixed Magnetic Bead Panel – BULK PACKAGED	HCYTMAG60PMX41BK

Antibody-Immobilized Magnetic Beads

Analyte	*Bead #*	*Cat. #*
EGF	12	HEGF-MAG
FGF-2	13	HCYFGF2-MAG
Eotaxin	14	HETXN-MAG
TGF-α	15	HCYTGFA-MAG
G-CSF	18	HGCSF-MAG
Flt-3L	19	HFLT3L-MAG
GM-CSF	20	HGMCSF-MAG
Fractalkine	21	HFKN-MAG
IFNα2	22	HIFNA2-MAG
IFNγ	25	HCYIFNG-MAG
GRO	26	HGR0-MAG
IL-10	27	HCYIL10-MAG
MCP-3	28	HMCP3-MAG
IL-12P40	29	HIL12P40-MAG
MDC	30	HMDC-MAG
IL-12P70	33	HIL12P70-MAG
PDGF-AA	34	HPDGFAA-MAG
IL-13	35	HIL13-MAG
PDGF-AB/BB	36	HPDGFBB-MAG
IL-15	37	HIL15-MAG
sCD40L	38	HCD40L-MAG
IL-17A	39	HIL17-MAG
IL-1RA	42	HIL1RA-MAG
IL-1α	44	HIL1A-MAG
IL-9	45	HIL9-MAG
IL-1β	46	HCYIL1B-MAG
IL-2	48	HIL2-MAG
IL-3	51	HIL3-MAG

Analyte	*Bead #*	*Cat. #*
IL-4	53	HIL4-MAG
IL-5	55	HIL5-MAG
IL-6	57	HCYIL6-MAG
IL-7	61	HIL7-MAG
IL-8	63	HCYIL8-MAG
IP-10	65	HIP10-MAG
MCP-1	67	HCYMCP1-MAG
MIP-1α	72	HMIP1A-MAG
MIP-1β	73	HMIP1B-MAG
RANTES	74	HCYRNTS-MAG
TNFα	75	HCYTNFA-MAG
TNFβ	76	HTNFB-MAG
VEGF	78	HCYVEGF-MAG
Premixed 29 Plex Beads		HCYPMX29-MAG
Premixed 38 Plex Beads		HCYPMX38-MAG

ORDERING INFORMATION

To place an order:

To assure the clarity of your custom kit order, please FAX the following information to our customer service department:

Include:

- Your name, telephone and/or fax number
- Customer account number
- Shipping and billing address
- Purchase order number
- Catalog number and description of product
- Quantity of kits
- Selection of MILLIPLEX® Analytes

FAX: (636) 441-8050

Toll-Free US: 1-800-MILLIPORE
781-533-8870

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

For European Customers:

To best serve our European customers in placing an order or obtaining additional information about MILLIPLEX® MAP products, please contact your multiplex specialist or sales representative or email our European Customer Service at:

Austria	AUCustomerService@merckgroup.com
Belgium	BENLCustomerService@merckgroup.com
Denmark	denmark@merckgroup.com
France	FRCustomerService@merckgroup.com
Finland	Asiakaspalvelu@merckgroup.com
Germany	GECustomerService@merckgroup.com
Ireland	IECustomerService@merckgroup.com
Italy	CSR-IT@merckgroup.com
Netherlands	BENLCustomerService@merckgroup.com
Norway	Norway@merckgroup.com
Spain	pedidos@merckgroup.com
Sweden	Kundservice@merckgroup.com
Switzerland	SZCustomerService@merckgroup.com
UK	UKCustomerService@merckgroup.com

ORDERING INFORMATION (continued)

Conditions of Sale

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

Material Safety Data Sheets (MSDS)

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

Technical Services

http://www.emdmillipore.com/techservices

To contact by phone

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

Outside North America, contact your local office
http://www.emdmillipore.com/offices

WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/mL Standard (Background)	400 pg/mL Standard	QC-2 Control									
B	0 pg/mL Standard (Background)	400 pg/mL Standard	QC-2 Control									
C	3.2 pg/mL Standard	2,000 pg/mL Standard	Sample 1									
D	3.2 pg/mL Standard	2,000 pg/mL Standard	Sample 1									
E	16 pg/mL Standard	10,000 pg/mL Standard	Sample 2									
F	16 pg/mL Standard	10,000 pg/mL Standard	Sample 2									
G	80 pg/mL Standard	QC-1 Control	Etc.									
H	80 pg/mL Standard	QC-1 Control										

Human Neurodegenerative Disease Magnetic Bead Panel 2

96-Well Plate Assay

Cat. # HNDG2MAG-36K

MILLIPLEX® MAP

HUMAN NEURODEGENERATIVE DISEASE MAGNETIC BEAD PANEL 2
96-Well Plate Assay

HNDG2MAG-36K

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

Human Neurodegenerative Disease Magnetic Bead Panel 2

INTRODUCTION

Neurodegenerative disease is a condition characterized by the deterioration of neurons or their myelin sheath over time in the brain and/or spinal cord. These neurons are responsible for such everyday activities as processing sensory information, making decisions, and controlling movement. Because these cells are not easily regenerated, excessive cumulative damage can lead to age-related diseases such as Alzheimer's and Parkinson's disease, as well as other conditions such as amyotrophic lateral sclerosis (ALS) and epilepsy. These disorders are devastating and expensive, both on a personal and global level, and as population demographics continue to change, a therapeutic solution is critical. Consequently, research is underway to identify biomarkers that will help scientists not only understand the pathogenesis of neurodegenerative disease, but also identify people with these disorders before the onset of symptoms and potentially provide new therapeutic tools.

Therefore, understanding neurobiology is fundamental to determining the pathogenesis of these devastating neurodegenerative diseases. Identification of key biomarkers and their accurate measurement is crucial. However, conventional methods, including RIAs and ELISAs, are not able to simultaneously measure multiple biomarkers with small sample volume. The Luminex®-based EMD Millipore's MILLIPLEX® MAP Human Neurodegenerative Magnetic Bead Panels will allow you to explore complexities of the nervous system and the pathobiology of disease.

MILLIPLEX® MAP offers the broadest selection of analytes across a wide range of disease states and species. Once the analytes of interest have been identified, you can rely on the quality that we build into each kit to produce results you can trust. In addition to the assay characteristics listed in the protocol, other performance criteria evaluated during the validation process include: cross-reactivity, dilution linearity, kit stability, and sample behavior (e.g. detectability and stability).

Each MILLIPLEX® MAP panel and kit includes:

- Quality controls (QCs) provided to qualify assay performance
- Comparison of standard (calibrator) and QC lots to a reference lot to ensure lot-to-lot consistency
- Optimized serum matrix to mimic native analyte environment
- Detection antibody cocktails designed to yield consistent analyte profiles within panel

In addition each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The **MILLIPLEX® MAP Human Neurodegenerative Disease Magnetic Bead Panel 2** thus enables you to focus on the therapeutic potential of neurobiological response. 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 Human Neurodegenerative Magnetic Bead Panel 2 is the most versatile system available for neurobiology research.

- MILLIPLEX MAP offers you the ability to choose any combination of analytes from our panel of 6 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 Human Neurodegenerative Magnetic Bead Panel 2 is to be used for the simultaneous quantification of the following 6 analytes in any combination**: CRP, α1-antitrypsin, PEDF, SAP, MIP-4, and Complement C4.** This kit may be used for the analysis of all above analytes in human serum, plasma, and cerebrospinal fluid samples.

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

Please read entire protocol before use.

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

PRINCIPLE

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

- Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- EMD Millipore provides three Luminex instruments to acquire and analyze data using two detection methods:
    - The Luminex analyzers Luminex 200™ and FLEXMAP 3D®, flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
    - The Luminex analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. EMD Millipore combines the streamlined data acquisition power of Luminex xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex instruments.

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

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze/thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8 °C

Reagents Supplied	Catalog Number	Volume	Quantity
Human Neurodegenerative Disease Panel 2 Standard	HNDG2-8036-2	Lyophilized	1 vial
Human Neurodegenerative Disease Panel 2 Quality Controls 1 and 2	HNDG2-6036-2	Lyophilized	2 vials
Bead Diluent	LBD	3.5 mL	1 bottle
Set of one 96-Well Plate with 2 sealers	----------	----------	1 plate 2 sealers
Assay Buffer	L-AB	30 mL	2 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	60 mL	1 bottle
Human Neurodegenerative Disease Panel 2 Detection Antibodies	HNDG2-1036-2	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE6	3.2 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

Human Neurodegenerative Disease Panel 2 Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable 6 Analytes (20X concentration, 200 µL)	
		Available	Cat. #
Anti-Human CRP Bead	19	✓	HCRP-MAG
Anti-Human α1-Antitrypsin Bead	28	✓	HA1AT-MAG
Anti-Human PEDF Bead	38	✓	HPEDF-MAG
Anti-Human SAP Bead	44	✓	HSAP-MAG
Anti-Human MIP-4 Bead	57	✓	HMIP4-MAG
Anti-Human Complement C4 Bead	63	✓	HCC4-MAG

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (EMD Millipore Catalog #SHEATHFLUID) or Luminex Drive Fluid (EMD Millipore 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 (BioTek® 405 LS and 405 TS, EMD Millipore Catalog #40-094, #40-095, #40-096, #40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog #40-285 or equivalent).

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

SAFETY PRECAUTIONS

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Note: See Full Labels of Hazardous components on next page.

Full Labels of Hazardous Components

Ingredient, Cat #		Full Label	
Human Neurodegenerative Disease Panel 2 Detection Antibodies	HNDG2-1036-2		**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.
Human Neurodegenerative Disease Panel 2 Quality Controls 1 & 2	HNDG2-6036-2		**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 Neurodegenerative Disease Panel 2 Standard	HNDG2-8036-2		**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.
Streptavidin-Phycoerythrin	L-SAPE6		**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.

TECHNICAL GUIDELINES

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

- For Research Use Only. Not for Use in Diagnostic Procedures.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock, which may be stored at $\leq$ -20°C for 1 month and at $\leq$ -70°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 or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc.

  For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.
- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells. If samples are diluted in Assay Buffer, use the Assay Buffer as matrix.

TECHNICAL GUIDELINES (continued)

- For serum/plasma samples that require further dilution beyond 1:2,000 (or CSF samples that require further dilution beyond 1:20), use the Assay Buffer provided in the kit.
- For cell/tissue homogenate, the final cell or tissue homogenate should be prepared in a buffer that has a neutral pH, contains minimal detergents or strong denaturing detergents, and has an ionic strength close to physiological concentration. Avoid debris, lipids, and cell/tissue aggregates. Centrifuge samples before use.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000xg. Remove serum and assay immediately or aliquot and store samples at ≤ -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 1:2,000 in the Assay Buffer provided in the kit. **Samples can be diluted using a two-step protocol. Step 1, add 5 µL serum to 495 µL Assay Buffer (i.e. 100-fold). Step 2, add 10 µL of the 100-fold diluted sample from Step 1 to another microfuge tube containing 190 µL Assay Buffer (i.e. 2,000-fold). Users may make similar dilutions using less sample volume to conserve Assay Buffer.** When further dilution beyond 1:2,000 is required, use Assay Buffer 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 1:2,000 in the Assay Buffer provided in the kit. **Samples can be diluted using a two-step protocol. Step 1, add 5 µL serum to 495 µL Assay Buffer (i.e. 100-fold). Step 2, add 10 µL of the 100-fold diluted sample from Step 1 to another microfuge tube containing 190 µL Assay Buffer (i.e. 2,000-fold). Users may make similar dilutions using less sample volume to conserve Assay Buffer.** When further dilution beyond 1:2,000 is required, use Assay Buffer as the diluent.

SAMPLE COLLECTION AND STORAGE (continued)

C. Preparation of CSF (cerebrospinal fluid):

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- ***Prior to the assay,*** CSF samples should be diluted 1:20 in the Assay Buffer. **Samples can be diluted by adding 5 µL CSF to 95 µL Assay Buffer (i.e. 20-fold). Users may make similar dilutions using less sample volume to conserve Assay Buffer.** If CSF samples require further dilution beyond 1:20, continue to use Assay Buffer as the sample diluent. Additional Assay Buffer can be purchased from EMD Millipore (EMD Millipore Catalog #L-AB). For diluted samples, multiply the final concentration of each analyte by the dilution factor.

D. 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 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

For individual vials of beads, sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 150 µL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent. 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 3 antibody-immobilized beads, add 150 µL from each of the 3 bead vials to the Mixing Bottle. Then add 2.55 mL Bead Diluent**.**

Example 2: When using 5 antibody-immobilized beads, add 150 µL from each of the 5 bead vials to the Mixing Bottle. Then add 2.25 mL Bead Diluent**.**

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. 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 with 540 mL deionized water. Store unused portion at 2-8°C for up to one month.

D. Preparation of Human Neurodegenerative Disease Panel 2 Standard

1.) Prior to use, reconstitute the Human Neurodegenerative Disease Panel 2 Standard with 250 µL deionized water (refer to table below for analyte concentration). Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. This will be used as Standard 7; the unused portion may be stored at ≤ -20°C for up to one month.

2). Preparation of Working Standards

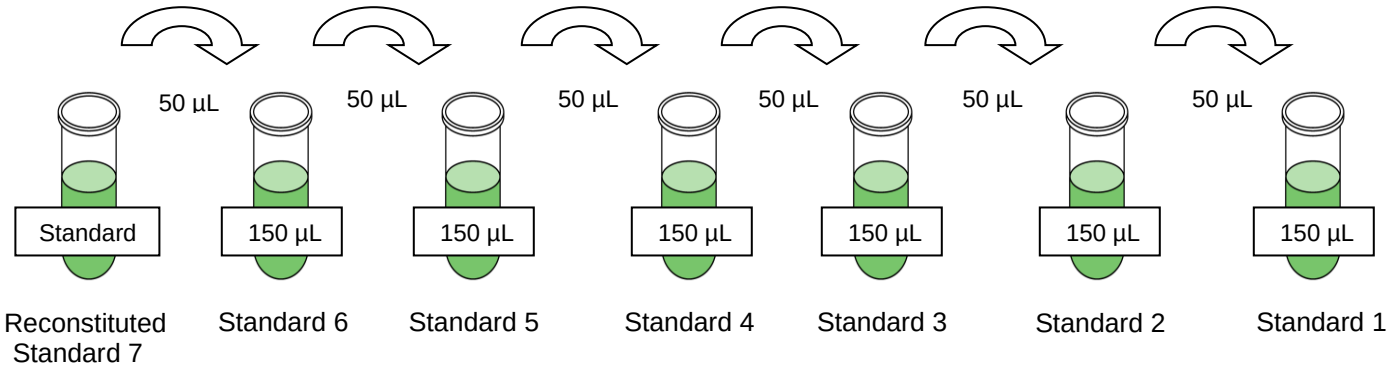
Label six polypropylene microfuge tubes as Standard 6, Standard 5, Standard 4, Standard 3, Standard 2, and Standard 1. Add 150 µL of Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 50 µL of the reconstituted Standard 7 to the Standard 6 tube, mix well and transfer 50 µL of the 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 and mix well. The 0 ng/mL standard (Background) will be Assay Buffer.

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

Standard (Tube #)	Volume of Deionized Water to Add	Volume of Standard To Add
Standard 7 (Reconstituted Standard)	250 µL	0

Standard (Tube #)	Volume of Assay Buffer to Add	Volume of Standard to Add
Standard 6	150 µL	50 µL of Standard 7
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 Standards



After dilution, each tube has the following concentrations for each analyte:

Standard	α1-Antitrypsin, Complement C4 (ng/mL)	SAP (ng/mL)	PEDF (ng/mL)	CRP (ng/mL)	MIP-4 (ng/mL)
Standard 1	0.244	0.122	0.049	0.012	0.005
Standard 2	0.977	0.488	0.195	0.049	0.020
Standard 3	3.906	1.953	0.781	0.195	0.078
Standard 4	15.625	7.813	3.125	0.781	0.313
Standard 5	62.5	31.25	12.5	3.125	1.25
Standard 6	250	125	50	12.5	5
Standard 7	1000	500	200	50	20

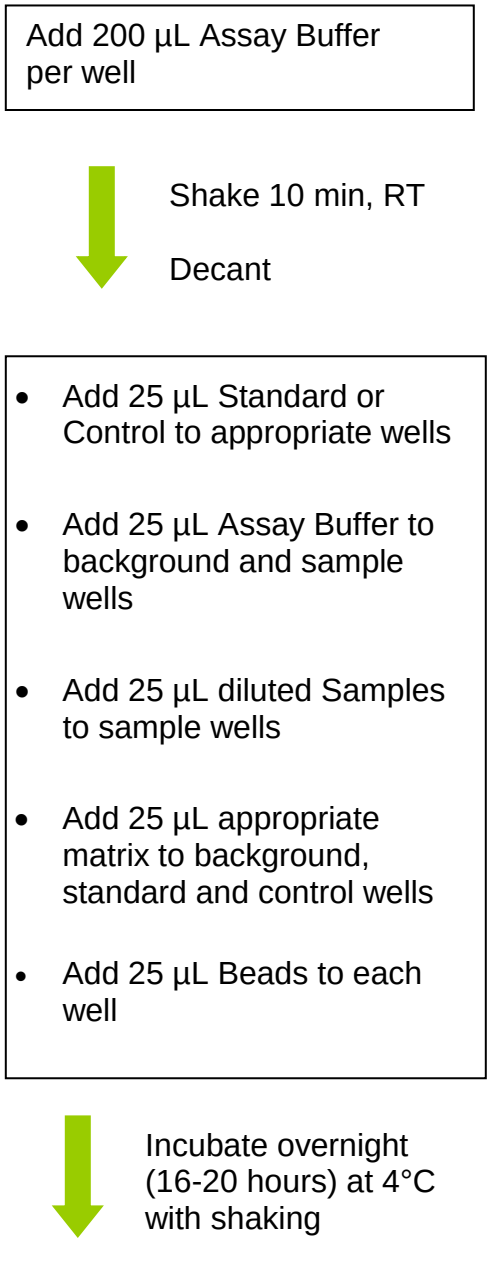
IMMUNOASSAY PROCEDURE

- 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 [Std 0 (Background), Standard 1, Standard 2, Standard 3, Standard 4, Standard 5, Standard 6, Standard 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 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 ng/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.

   A. When assaying 1:2,000 serum/ plasma or 1:20 diluted CSF, use the Assay Buffer provided in the kit as the matrix solution.

   B. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
6. Add 25 µL of Sample (tissue culture supernatant or diluted serum/plasma/CSF) into the appropriate wells.
7. Vortex Mixing Bottle and add 25 µL of the Mixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-20 hours) at 4°C.



9. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.

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

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

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

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

14. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.

15. Add 100 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 5 minutes.

16. Run plate on Luminex 200™, HTS, FLEXMAP 3D® or MAGPIX® with xPONENT® software.

17. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. Note: For diluted samples, multiply the calculated concentration by the dilution factor.)

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

Add 25 µL Detection Antibodies per well

Incubate 1 hour at RT

Do Not Aspirate

Add 25 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

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

Add 100 µL Sheath Fluid or Drive Fluid per well

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

PLATE WASHING

1.) Solid Plate

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

A.) Handheld magnet **(EMD Millipore Catalog #40-285)** - Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

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

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

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 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

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

These specifications are for the Luminex 200™, Luminex HTS, Luminex FLEXMAP 3D®, and Luminex MAGPIX® with xPONENT® software. Luminex instruments with other software (e.g. MasterPlex®, STarStation, LiquiChip, Bio-Plex Manager™, LABScan™ 100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex magnetic beads.

For magnetic bead assays, the Luminex 200™ and HTS instruments must be calibrated with the xPONENT® 3.1 compatible Calibration Kit (EMD Millipore Catalog #40-275) and performance verified with the Performance Verification Kit (EMD Millipore Catalog #40-276). The Luminex FLEXMAP 3D® instrument must be calibrated with the FLEXMAP 3D® Calibrator Kit (EMD Millipore Catalog #40-028) and performance verified with the FLEXMAP 3D® Performance Verification Kit (EMD Millipore Catalog #40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore Catalog #40-049) and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore Catalog #40-050).

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

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

EQUIPMENT SETTINGS (continued)

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

Events:	50, per bead	
Sample Size:	50 µL	
Gate Settings:	8,000 to 15,000	
Reporter Gain:	Default (low PMT)	
Time Out:	60 seconds	
Bead Set:	Customizable 6-Plex Beads	
	CRP	19
	α1-Antitrypsin	28
	PEDF	38
	SAP	44
	MIP-4	57
	Complement C4	63

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.emdmillipore.com using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Cross-Reactivity

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

Assay Sensitivities (minimum detectable concentrations, ng/mL)

Minimum Detectable Concentration (MinDC) is calculated using the MILLIPLEX® Analyst 5.1. 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 = 6 Assays)	
	MinDC (ng/mL)	MinDC+2SD (ng/mL)
CRP	0.0022	0.004
α1-Antitrypsin	0.0362	0.085
PEDF	0.008	0.016
SAP	0.009	0.023
MIP-4	0.0041	0.0049
Complement C4	0.0465	0.129

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 6 different assays.

Analyte	Overnight Protocol	
	Intra-assay %CV	Inter-assay %CV
CRP	<10	<15
α1-Antitrypsin	<10	<15
PEDF	<10	<15
SAP	<10	<15
MIP-4	<10	<15
Complement C4	<10	<15

ASSAY CHARACTERISTICS (continued)

Accuracy

Spike Recovery: The data represent mean percent recovery of spiked standards ranging from low, medium, and high concentration in 1:40,000 Assay Buffer diluted serum matrices (n=5).

Analyte	Overnight Protocol
	% Recovery in Serum Matrix
CRP	93
α1-Antitrypsin	92
PEDF	92
SAP	84
MIP-4	95
Complement C4	88

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 to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height to the kit solid plate using 1 alignment disc. For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated
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.
	Insufficient washes	Increase number of washes.

TROUBLESHOOTING GUIDE (continued)

Problem	Probable Cause	Solution
	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 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.
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.

TROUBLESHOOTING GUIDE (continued)

Problem	Probable Cause	Solution
	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.
	Samples have insoluble particles	Centrifuge samples just prior to assay setup and use supernatant.
	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 #
Human Neurodegenerative Disease Panel 2 Standard	HNDG2-8036-2
Human Neurodegenerative Disease Panel 2 Quality Controls	HNDG2-6036-2
Human Neurodegenerative Disease Panel 2 Detection Antibodies	HNDG2-1036-2
Streptavidin-Phycoerythrin	L-SAPE6
Assay Buffer	L-AB
Bead Diluent	LBD
Set of two 96-Well Plates with Sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Magnetic Beads

Analyte	*Bead #*	*Cat. #*
CRP	19	HCRP-MAG
α1-Antitrypsin	28	HA1AT-MAG
PEDF	38	HPEDF-MAG
SAP	44	HSAP-MAG
MIP-4	57	HMIP4-MAG
Complement C4	63	HCC4-MAG

ORDERING INFORMATION

To place an order:

To assure the clarity of your custom kit order, please FAX the following information to our customer service department:

Include:

- Your name, telephone and/or fax number
- Customer account number
- Shipping and billing address
- Purchase order number
- Catalog number and description of product
- Quantity of kits
- Selection of MILLIPLEX® Analytes

FAX: (636) 441-8050

Toll-Free US: (866) 441-8400
(636) 441-8400

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

For European Customers:

To best serve our European customers in placing an order or obtaining additional information about MILLIPLEX® MAP products, please contact your multiplex specialist or sales representative or email our European Customer Service at:

Austria	AUCustomerService@merckgroup.com
Belgium	BENLCustomerService@merckgroup.com
Denmark	denmark@merckgroup.com
France	FRCustomerService@merckgroup.com
Finland	Asiakaspalvelu@merckgroup.com
Germany	GECustomerService@merckgroup.com
Ireland	IECustomerService@merckgroup.com
Italy	CSR-IT@merckgroup.com
Netherlands	BENLCustomerService@merckgroup.com
Norway	Norway@merckgroup.com
Spain	pedidos@merckgroup.com
Sweden	Kundservice@merckgroup.com
Switzerland	SZCustomerService@merckgroup.com
UK	UKCustomerService@merckgroup.com

Conditions of Sale

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

Material Safety Data Sheets (MSDS)

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

Technical Services

http://www.emdmillipore.com/techservices

To contact by phone

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

Outside North America, contact your local office
http://www.emdmillipore.com/offices

WELL MAP

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

Human Neurodegenerative Disease Magnetic Bead Panel 3

96-Well Plate Assay

Cat. # HNDG3MAG-36K

MILLIPLEX® MAP

HUMAN NEURODEGENERATIVE DISEASE MAGNETIC BEAD PANEL 3
96-Well Plate Assay

HNDG3MAG-36K

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

Human Neurodegenerative Disease Magnetic Bead Panel 3

INTRODUCTION

Neurodegenerative disease is a condition characterized by the deterioration of neurons or their myelin sheath over time in the brain and/or spinal cord. These neurons are responsible for such everyday activities as processing sensory information, making decisions, and controlling movement. Because these cells are not easily regenerated, excessive cumulative damage can lead to age-related diseases such as Alzheimer's and Parkinson's disease, as well as other conditions such as amyotrophic lateral sclerosis (ALS) and epilepsy. These disorders are devastating and expensive, both on a personal and global level, and as population demographics continue to change, a therapeutic solution is critical. Consequently, research is underway to identify biomarkers that will help scientists not only understand the pathogenesis of neurodegenerative disease, but also identify people with these disorders before the onset of symptoms and potentially provide new therapeutic tools.

Therefore, understanding neurobiology is fundamental to determining the pathogenesis of these devastating neurodegenerative diseases. Identification of key biomarkers and their accurate measurement is crucial. However, conventional methods, including RIAs and ELISAs, are not able to simultaneously measure multiple biomarkers with small sample volume. The Luminex®-based Millipore's MILLIPLEX® MAP Human Neurodegenerative Magnetic Bead Panels will allow you to explore complexities of the nervous system and the pathobiology of disease.

To study neurodegenerative diseases, it might be necessary to screen different panels of specific proteins, which 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 comparable 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

In addition each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The **MILLIPLEX® MAP Human Neurodegenerative Disease Magnetic Bead Panel 3** thus enables you to focus on the therapeutic potential of neurobiological response. 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® MAP Human Neurodegenerative Magnetic Bead Panel 3 is the most versatile system available for neurobiology research.

- MILLIPLEX® MAP offers you the ability to choose any combination of analytes from our panel of 10 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 Human Neurodegenerative Magnetic Bead Panel 3 is to be used for the simultaneous quantification of the following 10 analytes in any combination**:** BDNF, Cathepsin D, sICAM-1, MPO, PDGF-AA, RANTES, NCAM, PDGF-AB/BB, sVCAM-1, and PAI-1(total). This kit may be used for the analysis of all above analytes in human serum, plasma, and cerebrospinal fluid samples.

For Research Use Only. Not for Use in Diagnostic Procedures.
Please read entire protocol before use.
It is important to use same assay incubation conditions throughout your study.

PRINCIPLE

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

- Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- EMD Millipore provides three Luminex instruments to acquire and analyze data using two detection methods:
    - The Luminex analyzers Luminex 200™ and FLEXMAP 3D®, flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
    - The Luminex analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. EMD Millipore combines the streamlined data acquisition power of Luminex xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex instruments.

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

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze/thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8 °C

Reagents Supplied	Catalog Number	Volume	Quantity
Human Neurodegenerative Disease Panel 3 Standard	HNDG3-8036-3	Lyophilized	1 vial
Human Neurodegenerative Disease Panel 3 Quality Controls 1 and 2	HNDG3-6036-3	Lyophilized	2 vials
Bead Diluent	MXBD-3	3.5 mL	1 bottle
Set of one 96-Well Plate with 2 sealers	----------	----------	1 plate 2 sealers
Assay Buffer	L-AB	30 mL	2 bottles
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	60mL	1 bottle
Human Neurodegenerative Disease Panel 3 Detection Antibodies	HNDG3-1036-3	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE4	3.2 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

Human Neurodegenerative Disease Panel 3 Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex® Magnetic Bead Region	Customizable 10 Analytes (20X concentration, 200 µL)	
		Available	Cat. #
Anti-Human BDNF Bead	15	✓	RBDNF-MAG
Anti-Human Cathepsin D Bead	22	✓	HCTHPSND-MAG
Anti-Human sICAM-1 Bead	35	✓	HSICM1-MAG
Anti-Human MPO Bead	37	✓	HMP0-MAG
Anti-Human PDGF-AA Bead	42	✓	HNDPDGFAA-MAG
Anti-Human RANTES Bead	52	✓	HRNTS-MAG
Anti-Human NCAM Bead	55	✓	HNCAM-MAG
Anti-Human PDGF-AB/BB Bead	61	✓	HNDPDGFBB-MAG
Anti-Human sVCAM-1 Bead	74	✓	HSVCM1-MAG
Anti-Human PAI-1 (total) Bead	76	✓	HTPAI1-MAG

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (EMD Millipore Catalog #SHEATHFLUID) or Luminex Drive Fluid (EMD Millipore 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 (BioTek® 405 LS and 405 TS, EMD Millipore Catalog #40-094, #40-095, #40-096, #40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog #40-285 or equivalent).

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

SAFETY PRECAUTIONS

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

See next page for full hazardous labels.

Full hazardous labels for components in this kit:

Ingredient, Cat #		Full Label	
Human Neurodegenerative Disease Panel 3 Detection Antibodies	HNDG3-1036-3		**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.
Human Neurodegenerative Disease Panel 3 Quality Controls 1 and 2	HNDG3-6036-3		**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 Neurodegenerative Disease Panel 3 Standard	HNDG3-8036-3		**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.
Bead Diluent	MXBD-3		**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	L-SAPE4		**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.
Wash Buffer	L-WB		**Warning.** May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.

TECHNICAL GUIDELINES

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

- For Research Use Only. Not for Use in Diagnostic Procedures.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at $\leq$ -20°C for 1 month and at $\leq$ -70°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 or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc.
- For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated. For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells. If samples are diluted in assay buffer, use the assay buffer as matrix.

TECHNICAL GUIDELINES (continued)

- For serum/plasma samples that require further dilution beyond 1:100 (or CSF samples that require further dilution), use the assay buffer provided in the kit.
- For cell/tissue homogenate, the final cell or tissue homogenate should be prepared in a buffer that has a neutral pH, contains minimal detergents or strong denaturing detergents, and has an ionic strength close to physiological concentration. Avoid debris, lipids, and cell/tissue aggregates. Centrifuge samples before use.
- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000xg. Remove serum and assay immediately or aliquot and store samples at ≤ -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 1:100 in the assay buffer provided in the kit. **Samples can be diluted by adding 5 µL serum to 495 µL Assay Buffer (i.e. 100-fold). Users may make similar dilutions using less sample volume to conserve Assay Buffer.** When further dilution beyond 1:100 is required, use assay buffer 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 1:100 in the assay buffer provided in the kit. **Samples can be diluted by adding 5 µL plasma to 495 µL Assay Buffer (i.e. 100-fold). Users may make similar dilutions using less sample volume to conserve Assay Buffer. Users may make similar dilutions using less sample volume to conserve Assay Buffer.** When further dilution beyond 1:100 is required, use assay buffer as the diluent.

SAMPLE COLLECTION AND STORAGE (continued)

C. Preparation of CSF (cerebrospinal fluid):

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- CSF samples do not required dilution. If CSF samples require dilution, use Assay Buffer as the sample diluent. Additional Assay Buffer can be purchased from EMD Millipore (EMD Millipore Catalog # L-AB). For diluted samples, multiply the final concentration of each analyte by the dilution factor.

D. 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 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

For individual vials of beads, sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 150 µL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent. 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 3 antibody-immobilized beads, add 150 µL from each of the 3 bead vials to the Mixing Bottle. Then add 2.55 mL Bead Diluent**.**

Example 2: When using 5 antibody-immobilized beads, add 150 µL from each of the 5 bead vials to the Mixing Bottle. Then add 2.25 mL Bead Diluent**.**

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

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. 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 with 540 mL deionized water. Store unused portion at 2-8°C for up to one month.

D. Preparation of Human Neurodegenerative Disease Panel 3 Standard

1.) Prior to use, reconstitute the Human Neurodegenerative Disease Panel 3 Standard with 250 µL deionized water (refer to table below for analyte concentration). Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. This will be used as Standard 7; the unused portion may be stored at ≤ -20°C for up to one month.

2). Preparation of Working Standards

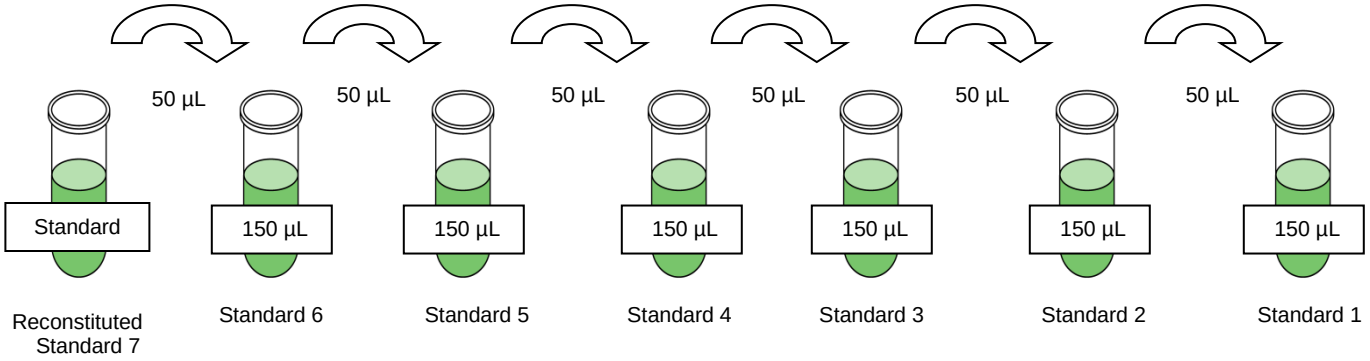
Label six polypropylene microfuge tubes as Standard 6, Standard 5, Standard 4, Standard 3, Standard 2, and Standard 1. Add 150 µL of Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 50 µL of the reconstituted Standard 7 to the Standard 6 tube, mix well and transfer 50 µL of the 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 and mix well. The 0 pg/mL standard (Background) will be Assay Buffer.

Standard (Tube #)	Volume of Deionized Water to Add	Volume of Standard To Add
Standard 7 (Reconstituted Standard)	250 µL	0

Standard (Tube #)	Volume of Assay Buffer to Add	Volume of Standard to Add
Standard 6	150 µL	50 µL of Standard 7
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)

Preparation of Standards



After dilution, each tube has the following concentrations for each analyte:

Standard	sVCAM-1 (pg/mL)	sICAM-1, MPO, Cathepsin D, PDGF-AB/BB, NCAM (pg/mL)	BDNF, PDGF-AA, RANTES, PAI-1(total) (pg/mL)
Standard 1	61	24	2
Standard 2	244	98	10
Standard 3	977	391	39
Standard 4	3,906	1,563	156
Standard 5	15,625	6,250	625
Standard 6	62,500	25,000	2,500
Standard 7	250,000	100,000	10,000

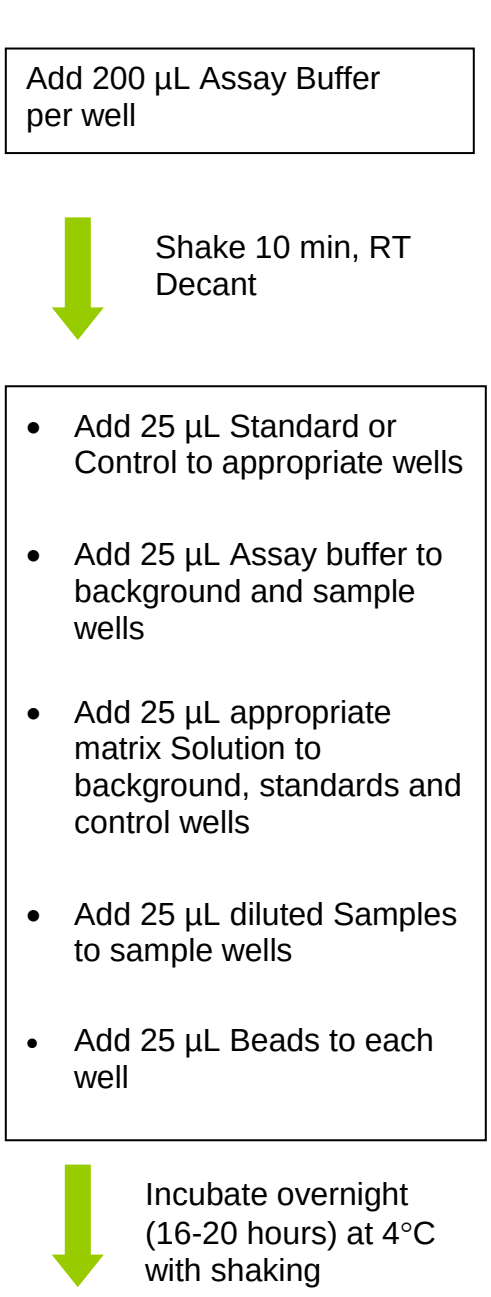
IMMUNOASSAY PROCEDURE

- 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 [Std 0 (Background), Standard 1, Standard 2, Standard 3, Standard 4, Standard 5, Standard 6, Standard 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 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of each Standard or Control into the appropriate wells. Assay Buffer should be used for 0 pg/mL standard (Background).
4. Add 25 µL of Assay Buffer to the sample wells.
5. Add 25 µL of appropriate matrix solution to the background, standards, and control wells.

   A. When assaying 1:100 serum/ plasma or undiluted CSF, use the Assay Buffer provided in the kit as the matrix solution.

   B. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
6. Add 25 µL of Sample (tissue culture supernatant or diluted serum/plasma, or undiluted CSF) into the appropriate wells.
7. Vortex Mixing Bottle and add 25 µL of the Mixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight (16-20 hours) at 4°C.



9. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.

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

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

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

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

14. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.

15. Add 100 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 5 minutes.

16. Run plate on Luminex 200™, HTS, FLEXMAP 3D® or MAGPIX® with xPONENT® software.

17. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. Note: For diluted samples, multiply the calculated concentration by the dilution factor.)

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

Add 25 µL Detection Antibodies per well

Incubate 1 hour at RT

Do Not Aspirate

Add 25 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

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

Add 100 µL Sheath Fluid or Drive Fluid per well

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

PLATE WASHING

1.) Solid Plate

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

A.) Handheld magnet **(EMD Millipore Catalog #40-285)** - Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

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

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

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

EQUIPMENT SETTINGS

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

These specifications are for the Luminex 200™, Luminex HTS, Luminex FLEXMAP 3D®, and Luminex MAGPIX® with xPONENT® software. Luminex instruments with other software (e.g. MasterPlex®, STarStation, LiquiChip, Bio-Plex Manager™, LABScan™ 100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex magnetic beads.

For magnetic bead assays, the Luminex 200™ and HTS instruments must be calibrated with the xPONENT® 3.1 compatible Calibration Kit (EMD Millipore Catalog #40-275) and performance verified with the Performance Verification Kit (EMD Millipore Catalog #40-276). The Luminex FLEXMAP 3D® instrument must be calibrated with the FLEXMAP 3D® Calibrator Kit (EMD Millipore Catalog #40-028) and performance verified with the FLEXMAP 3D® Performance Verification Kit (EMD Millipore Catalog #40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore Catalog #40-049) and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore Catalog #40-050).

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

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

EQUIPMENT SETTINGS (continued)

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

Events:	50, per bead	
Sample Size:	50 µL	
Gate Settings:	8,000 to 15,000	
Reporter Gain:	Default (low PMT)	
Time Out:	60 seconds	
Bead Set:	Customizable 10-Plex Beads	
	BDNF	15
	Cathepsin D	22
	sICAM-1	35
	MPO	37
	PDGF-AA	42
	RANTES	52
	NCAM	55
	PDGF-AB/BB	61
	sVCAM-1	74
	PAI-1 (total)	76

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.emdmillipore.com using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Cross-Reactivity

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

Assay Sensitivities (minimum detectable concentrations)

Minimum Detectable Concentration (MinDC) is calculated using the MILLIPLEX Analyst 5.1. 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 = 6 Assays)	
	MinDC (pg/mL)	MinDC+2SD (pg/mL)
BDNF	0.23	0.47
Cathepsin D	8.08	23.91
sICAM-1	6.29	16.13
MPO	200	550
PDGF-AA	0.22	0.51
RANTES	1.20	1.72
NCAM	4.81	13.48
PDGF-AB/BB	3.83	6.87
sVCAM-1	6.44	12.24
PAI-1 (total)	0.48	0.92

Precision

Intra-assay precision is generated from the mean of the %CVs from 16 reportable results across two different concentrations of analytes in a single assay. Inter-assay precision is generated from the mean of the %CVs across two different concentrations of analytes across 10 different assays.

Analyte	Overnight Protocol	
	Intra-assay %CV	Inter-assay %CV
BDNF	5.4	5.3
Cathepsin D	5.9	12.9
sICAM-1	5.7	5.7
MPO	5.1	8.9
PDGF-AA	5.9	12.1
RANTES	4.0	4.7
NCAM	3.5	4.9
PDGF-AB/BB	6.0	5.3
sVCAM-1	2.8	7.3
PAI-1 (total)	3.3	5.7

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=5).

Analyte	Overnight Protocol
	% Recovery in Serum Matrix
BDNF	114
Cathepsin D	111
sICAM-1	108
MPO	100
PDGF-AA	123
RANTES	108
NCAM	103
PDGF-AB/BB	137
sVCAM-1	104
PAI-1 (total)	92

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	See above
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.
	Insufficient washes	Increase number of washes.
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 (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 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 (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 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 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 pipette tips that are used for reagent additions and that pipette tip does not touch reagent in plate.

FOR FILTER PLATES ONLY		
Problem	**Probable Cause**	**Solution**
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.
	Samples have insoluble particles	Centrifuge samples just prior to assay setup and use supernatant.
	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 #
Human Neurodegenerative Disease Panel 3 Standard	HNDG3-8036-3
Human Neurodegenerative Disease Panel 3 Quality Controls	HNDG3-6036-3
Human Neurodegenerative Disease Panel 3 Detection Antibodies	HNDG3-1036-3
Streptavidin-Phycoerythrin	L-SAPE4
Assay Buffer	L-AB
Bead Diluent	MXBD-3
Set of two 96-Well Plates with Sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Magnetic Beads

Analyte	*Bead #*	*Cat. #*
BDNF	15	RBDNF-MAG
Cathepsin D	22	HCTHPSND-MAG
sICAM-1	35	HSICM1-MAG
MPO	37	HMP0-MAG
PDGF-AA	42	HNDPDGFAA-MAG
RANTES	52	HRNTS-MAG
NCAM	55	HNCAM-MAG
PDGF-AB/BB	61	HNDPDGFBB-MAG
sVCAM-1	74	HSVCM1-MAG
PAI-1 (total)	76	HTPAI1-MAG

ORDERING INFORMATION

To place an order:

To assure the clarity of your custom kit order, please FAX the following information to our customer service department:

Include:

- Your name, telephone and/or fax number
- Customer account number
- Shipping and billing address
- Purchase order number
- Catalog number and description of product
- Quantity of kits
- Selection of MILLIPLEX® Analytes

FAX: (636) 441-8050

Toll-Free US: (866) 441-8400
(636) 441-8400

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

For European Customers:

To best serve our European customers in placing an order or obtaining additional information about MILLIPLEX® MAP products, please contact your multiplex specialist or sales representative or email our European Customer Service at:

Austria	AUCustomerService@merckgroup.com
Belgium	BENLCustomerService@merckgroup.com
Denmark	denmark@merckgroup.com
France	FRCustomerService@merckgroup.com
Finland	Asiakaspalvelu@merckgroup.com
Germany	GECustomerService@merckgroup.com
Ireland	IECustomerService@merckgroup.com
Italy	CSR-IT@merckgroup.com
Netherlands	BENLCustomerService@merckgroup.com
Norway	Norway@merckgroup.com
Spain	pedidos@merckgroup.com
Sweden	Kundservice@merckgroup.com
Switzerland	SZCustomerService@merckgroup.com
UK	UKCustomerService@merckgroup.com

ORDERING INFORMATION (continued)

Conditions of Sale

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

Material Safety Data Sheets (MSDS)

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

Technical Services

http://www.emdmillipore.com/techservices

To contact by phone

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

Outside North America, contact your local office
http://www.emdmillipore.com/offices

WELL MAP

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

Human Soluble Cytokine Receptor Magnetic Bead Panel

96-Well Plate Assay

Cat. # HSCRMAG-32K
HSCRMAG32KPX14

MILLIPLEX® MAP

HUMAN SOLUBLE CYTOKINE RECEPTOR MAGNETIC BEAD PANEL
96-Well Plate Assay

HSCRMAG-32K or
HSCRMAG32KPX14

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Human Soluble Cytokine Receptor Magnetic Bead Panel

INTRODUCTION

Cytokine receptors constitute an integral part of the cytokine biology. Like cytokines, cytokine receptors are involved in normal physiological and pathological processes of almost all disease states. Soluble cytokine receptors naturally arise from genes encoding membrane- bound receptors or are direct derivatives of the receptors themselves. The discovery that soluble cytokine receptors are involved in regulating excessive inflammatory responses and modulating immune events has stimulated significant research interest in their potential role as immunotherapeutic agents. Many of these soluble cytokine receptors have the ability to inhibit the binding and biological activity of their cytokine ligands, making them very specific cytokine antagonists.

Designed for the simultaneous analysis of multiple biomarkers, the Human Soluble Cytokine Receptor Panel provides important tools for the study of inflammatory and immune responses—evidence of EMD Millipore's commitment to innovation and providing you with the best tools to do your best work.

MILLIPLEX® MAP offers the broadest selection of analytes across a wide range of disease states and species. Once the analytes of interest have been identified, you can rely on the quality that we build into each kit to produce results you can trust. In addition to the assay characteristics listed in the protocol, other performance criteria evaluated during the validation process include: cross-reactivity, dilution linearity, kit stability, and sample behavior (e.g. detectability and stability).

Each MILLIPLEX® MAP panel and kit includes:

- Quality controls (QCs) provided to qualify assay performance
- Comparison of standard (calibrator) and QC lots to a reference lot to ensure lot-to-lot consistency
- Optimized serum matrix to mimic native analyte environment
- Detection antibody cocktails designed to yield consistent analyte profiles within panel

In addition each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The **MILLIPLEX® MAP** Human Soluble Cytokine Receptor Magnetic Bead Panel thus enables you to focus on the therapeutic potential of soluble cytokine receptors. 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® MAP Human Soluble Cytokine Receptor Magnetic Bead Panel is part of the most versatile system available for cytokine receptor research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically validate and build the most comprehensive library available for protein detection and quantitation.

- MILLIPLEX® MAP offers you:
    - The ability to select a 14-plex or premixed option or
    - The ability to choose any combination of analytes from our panel of 14 analytes to design a custom kit that better meets your needs.
    - A convenient "all-in-one" box format that gives you the assurance that you will have all the necessary reagents you need to run your assay.

EMD Millipore's MILLIPLEX® MAP Human Soluble Cytokine Receptor Magnetic Bead Panel is to be used for the simultaneous quantification of any or all of the following analytes in human tissue/cell lysate and culture supernatant samples and serum or plasma samples: soluble CD30 (sCD30, sTNFRSF8), soluble Epidermal Growth Factor Receptor (sEGFR), soluble gp130 (sgp130), soluble Interleukin-1 Receptor Type I (sIL-1RI, sCD121a), soluble Interleukin-1 Receptor Type II (sIL-1RII, sCD121b), soluble Interleukin-2 Receptor alpha (sIL-2Rα, CD25), soluble Interleukin-4 Receptor (sIL-4R, CD124), soluble Interleukin-6 Receptor (sIL-6R, CD126), soluble Receptor for Advanced Glycation Endproducts (sRAGE), soluble Tumor Necrosis Factor Receptor I (sTNFRI, TNFRSF1A), soluble Tumor Necrosis Factor Receptor II (sTNFRII, TNFRSF1B), soluble Vascular Endothelial Growth Factor Receptor 1 (sVEGFR1, sFlt-1), soluble Vascular Endothelial Growth Factor Receptor 2 (sVEGFR2, sFlk-1, sKDR), and soluble Vascular Endothelial Growth Factor Receptor 3 (sVEGFR3, sFlt-4).

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Please read entire protocol before use.

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

PRINCIPLE

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

- Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- EMD Millipore provides three Luminex instruments to acquire and analyze data using two detection methods:
    - The Luminex analyzers Luminex 200™ and FLEXMAP 3D®, flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
    - The Luminex analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. EMD Millipore combines the streamlined data acquisition power of Luminex xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex instruments.

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

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze/thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

Reagents Supplied	Catalog Number	Volume	Quantity
Human Soluble Cytokine Receptor Standard	HSCR-8032	Lyophilized	1 vial
Human Soluble Cytokine Receptor Quality Controls 1 and 2	HSCR-6032	Lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	HSCR-SM	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	60 mL	1 bottle
Human Soluble Cytokine Receptor Detection Antibodies	HSCRMG-1032	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE3	3.2 mL	1 bottle
Bead Diluent (not provided with premixed panel)	LBD	3.5 mL	1 bottle
Mixing Bottle (not provided with premixed panel)	----------	----------	1 bottle

Human Soluble Cytokine Receptor Antibody-Immobilized Premixed Magnetic Beads:

Premixed 13-plex Beads + EGFR	HSCRPMX13-MAG, HEGFR-MAG	3.5 mL, 90 µL	1 bottle + 1 bead vial

Included Human Soluble Cytokine Receptor Antibody-Immobilized Magnetic Beads are dependent on customizable selection of analytes within the panel (see next page).

REAGENTS SUPPLIED (continued)

Human Soluble Cytokine Receptor Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable 14 Analytes (50X concentration, 90 µL) Available	Cat. #	13-Plex Magnetic Premixed Beads + HEGFR-MAG
Anti – sCD30 Bead	12	✓	HCD30-MAG	✓
Anti – sEGFR Bead	14	✓	HEGFR-MAG	✓ Single Vial
Anti – sgp130 Bead	18	✓	HGP130-MAG	✓
Anti – sIL-1RI Bead	20	✓	HIL1R1-MAG	✓
Anti – sIL-1RII Bead	22	✓	HIL1R2-MAG	✓
Anti – sIL-2Rα Bead	33	✓	HSIL2RA-MAG	✓
Anti – sIL-4R Bead	35	✓	HIL4R-MAG	✓
Anti – sIL-6R Bead	37	✓	HIL6R-MAG	✓
Anti – sRAGE Bead	39	✓	HSRAGE-MAG	✓
Anti – sTNFRI Bead	51	✓	HTNFR1-MAG	✓
Anti – sTNFRII Bead	53	✓	HTNFR2-MAG	✓
Anti – sVEGFR1 Bead	55	✓	HVEGFR1-MAG	✓
Anti – sVEGFR2 Bead	57	✓	HVEGFR2-MAG	✓
Anti – sVEGFR3 Bead	61	✓	HVEGFR3-MAG	✓

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (EMD Millipore Catalog #SHEATHFLUID) or Luminex Drive Fluid (EMD Millipore 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 (BioTek® 405 LS and 405 TS, EMD Millipore Catalog #40-094, #40-095, #40-096, #40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog #40-285 or equivalent).

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

SAFETY PRECAUTIONS

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Note: See Full Labels of Hazardous components on next page.

Full Labels of Hazardous Components:

Ingredient, Cat #		Full Label	
Human Soluble Cytokine Receptor Quality Control 1 & 2	HSCR-6032		**Warning.** Harmful if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment.
Human Soluble Cytokine Receptor Standard	HSCR-8032		**Warning.** Harmful if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment.
Human Soluble Cytokine Receptor Detection Antibodies	HSCRMG-1032		**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.
Serum Matrix	HSCR-SM		**Warning.** Harmful if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment.
Streptavidin-Phycoerythrin	L-SAPE3		**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.

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 with the appropriate diluent and repeat the assay.
- Mix only required amount of beads prior to assay setup. Discard any unused premixed beads.
- During the preparation of the standard curve, make certain to mix the higher concentration well before making the next dilution. Use a new tip with each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some analytes.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.
- When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc.

  For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.
- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells. If samples are diluted in assay buffer, use the assay buffer as matrix.

TECHNICAL GUIDELINES (continued)

- For serum/plasma samples that require further dilution beyond 1:5, use the Serum Matrix 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.
   - Serum samples should be diluted 1:5 in the Serum Matrix provided in the kit. For example, in a tube, 20 µL of serum may be combined with 80 µL Serum Matrix. When further dilution beyond 1:5 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 1:5 in the Serum Matrix provided in the kit. For example, in a tube, 20 µL of plasma may be combined with 80 µL Serum Matrix. When further dilution beyond 1:5 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. 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 1:5 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

A. Preparation of Antibody-Immobilized Beads

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

To prepare 14-plex premixed beads, add 70µL of HEGFR-MAG bead to the 13-plex premixed beads. Mix well before use.

For individual vials of beads, sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 60 µL from each antibody-bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent. Vortex the mixed beads well. Discard any unused premixed beads. (Note: Due to the composition of magnetic beads, you may notice a slight color in the bead solution. This does not affect the performance of the beads or the kit.)

Example 1: When using 3 antibody-immobilized beads, add 60 µL from each of the 3 bead vials to the Mixing Bottle. Then add 2.82 mL Bead Diluent.

Example 2: When using 11 antibody-immobilized beads, add 60 µL from each of the 11 bead vials to the Mixing Bottle. Then add 2.34 mL Bead Diluent.

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. 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 with 540 mL deionized water. Store the unused portion at 2-8°C for up to one month.

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

D. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

Add 5.0 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 Human Soluble Cytokine Receptor Standard

1.) Prior to use, reconstitute the Human Soluble Cytokine Receptor Panel Standard with 250 µL deionized water to give [refer to table below for analyte concentrations if using multiple standard concentrations]. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. This will be used as Standard 7; the unused portion may be stored at ≤ -20°C for up to one month.

2). Preparation of Working Standards

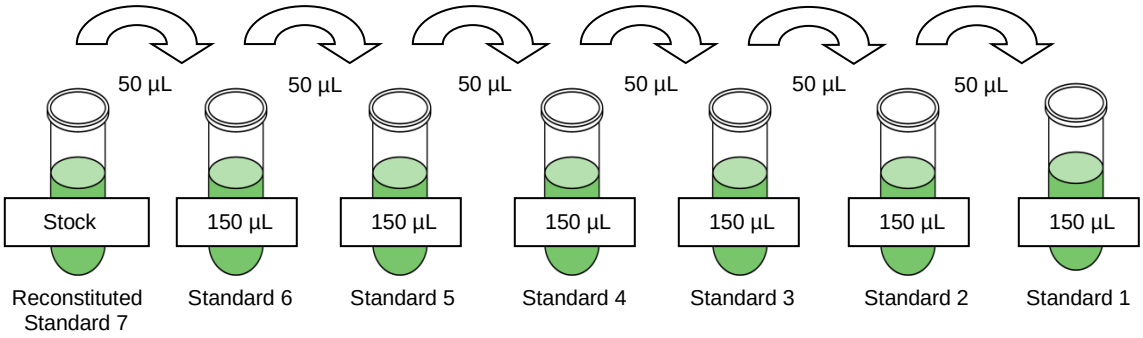
Label six polypropylene microfuge tubes Standard 6, Standard 5, Standard 4, Standard 3, Standard 2 and Standard 1. Add 150 µL of Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 50 µL of the reconstituted standard (Standard 7) to the Standard 6 tube, mix well and transfer 50 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 Assay Buffer.

Standard (Tube #)	Volume of Deionized Water to Add	Volume of Standard to Add
Standard 7 (reconstituted standard)	250 µL	0

Standard (Tube #)	Volume of Assay Buffer to Add	Volume of Standard to Add
Standard 6	150 µL	50 µL of Standard 7
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)

Preparation of Standards

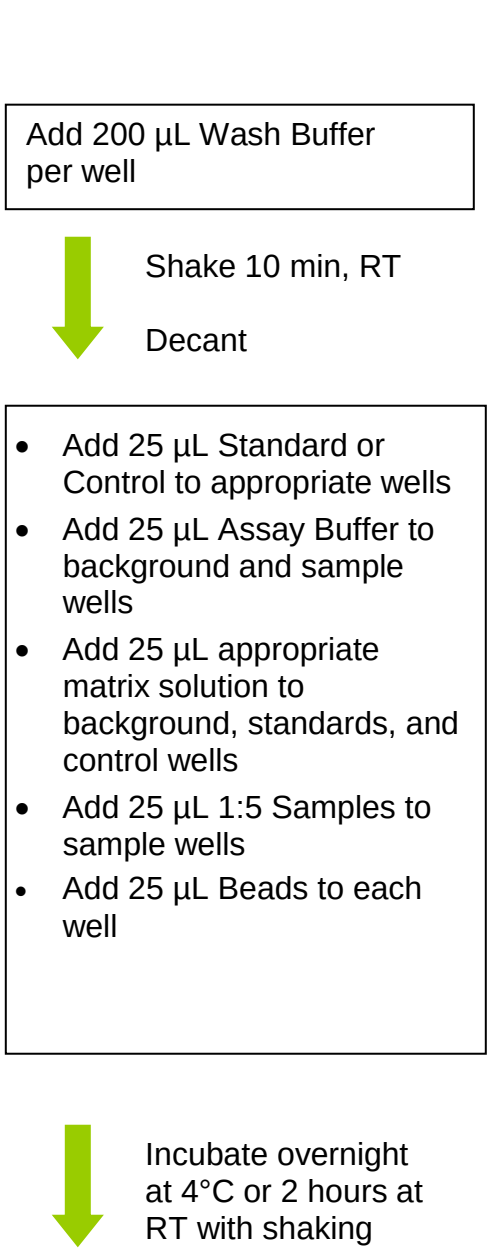


Standard	sIL-4R, sIL-6R sTNFRI, sTNFRII, sRAGE (pg/mL)	sCD30, sgp130, sIL-1RI, sIL-2Rα (pg/mL)	sEGFR, sIL-1RII, sVEGFR1, sVEGFR2, sVEGFR3 (pg/mL)
Standard 7	50,000	100,000	500,000
Standard 6	12,500	25,000	125,000
Standard 5	3,125	6,250	31,250
Standard 4	781.3	1,562.5	7,812.5
Standard 3	195.3	390.6	1,953.1
Standard 2	48.8	97.7	488.3
Standard 1	12.2	24.4	122.1

IMMUNOASSAY PROCEDURE

- Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines.
- Allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Diagram the placement of Standards [0 (Background), [Standard 1 through 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 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 diluted 1:5 into the appropriate wells.
7. Vortex Mixing Bottle and add 25 µL of the Mixed or Premixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker overnight 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.



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

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

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

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

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

14. Gently remove well contents and wash plate 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 on a plate shaker for 5 minutes.

16. Run plate on Luminex 200™, HTS, FLEXMAP 3D® or MAGPIX® with xPONENT® software.

17. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. (Note: For diluted samples, multiply the calculated concentration by the dilution factor.)

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

Add 25 µL Detection Antibodies per well

Incubate 1 hour at RT

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 handheld magnet or magnetic plate washer.

A.) Handheld magnet **(EMD Millipore Catalog #40-285)** - Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

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

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

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 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

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

These specifications are for the Luminex 200™, Luminex HTS, Luminex FLEXMAP 3D®, and Luminex MAGPIX® with xPONENT® software. Luminex instruments with other software (e.g. MasterPlex®, STarStation, LiquiChip, Bio-Plex Manager™, LABScan™ 100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex magnetic beads.

For magnetic bead assays, the Luminex 200™ and HTS instruments must be calibrated with the xPONENT® 3.1 compatible Calibration Kit (EMD Millipore Catalog #40-275) and performance verified with the Performance Verification Kit (EMD Millipore Catalog #40-276). The Luminex FLEXMAP 3D® instrument must be calibrated with the FLEXMAP 3D® Calibrator Kit (EMD Millipore Catalog #40-028) and performance verified with the FLEXMAP 3D® Performance Verification Kit (EMD Millipore Catalog #40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore Catalog #40-049) and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore Catalog #40-050).

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

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

EQUIPMENT SETTINGS (continued)

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

Events:	50, per bead	
Sample Size:	100 µL	
Gate Settings:	8,000 to 15,000	
Reporter Gain:	Default (low PMT)	
Time Out:	60 seconds	
Bead Set:	Customizable 14-Plex Beads	
	sCD30	12
	sEGFR	14
	sgp130	18
	sIL-1RI	20
	sIL-1RII	22
	sIL-2Rα	33
	sIL-4R	35
	sIL-6R	37
	sRAGE	39
	sTNFRI	51
	sTNFRII	53
	sVEGFR1	55
	sVEGFR2	57
	sVEGFR3	61

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.emdmillipore.com using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Cross-Reactivity

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

Assay Sensitivities (minimum detectable concentrations, pg/mL)

Minimum Detectable Concentration (MinDC) is calculated using MILLIPLEX® Analyst 5.1. 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 = 5 Assays)		2 Hour Protocol (n = 3 Assays)	
	MinDC (pg/mL)	MinDC+2SD (pg/mL)	MinDC (pg/mL)	MinDC+2SD (pg/mL)
sCD30	7	14.7	12	28.1
sEGFR	42	60.7	47	68.4
sgp130	6	6.9	6	8.5
sIL-1RI	21	22	9	15.9
sIL-1RII	115	115.6	37	47.4
sIL-2Rα	11	20.9	6	6.6
sIL-4R	14	17.9	10	22.6
sIL-6R	9	14.1	5	11.4
sRAGE	3	3.8	5	9.6
sTNFRI	12	14.3	6	14.6
sTNFRII	8	19.2	11	18.6
sVEGFR1	111	208.4	96	248
sVEGFR2	71	116.4	94	133.8
sVEGFR3	47	121.9	66	137.4

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 4 different assays.

Analyte	Overnight Protocol		2 Hour Protocol
	Intra-assay %CV	Inter-assay %CV	Intra-assay %CV
sCD30	< 10	< 15	< 10
sEGFR	< 10	< 15	< 10
sgp130	< 10	< 15	< 10
sIL-1RI	< 10	< 15	< 10
sIL-1RII	< 10	< 15	< 10
sIL-2Rα	< 10	< 15	< 10
sIL-4R	< 10	< 15	< 10
sIL-6R	< 10	< 15	< 10
sRAGE	< 10	< 15	< 10
sTNFRI	< 10	< 15	< 10
sTNFRII	< 10	< 15	< 10
sVEGFR1	< 10	< 15	< 10
sVEGFR2	< 10	< 15	< 10
sVEGFR3	< 10	< 15	< 10

Accuracy

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

Analyte	Overnight Protocol
	% Recovery in Serum Matrix
sCD30	101
sEGFR	101
sgp130	103
sIL-1RI	103
sIL-1RII	96
sIL-2Rα	91
sIL-4R	104
sIL-6R	103
sRAGE	104
sTNFRI	97
sTNFRII	99
sVEGFR1	97
sVEGFR2	101
sVEGFR3	96

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 flushes, back flushes 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 to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height to the kit solid plate using 1 alignment disc. For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.
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 (e.g. interleukin modified tissue culture medium).
	Insufficient washes	Increase number of washes.
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 (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 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.
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 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.

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.
	Samples have insoluble particles	Centrifuge samples just prior to assay set-up and use supernatant.
	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 #**
Human Soluble Cytokine Receptor Standard	HSCR-8032
Human Soluble Cytokine Receptor Quality Controls 1 and 2	HSCR-6032
Serum Matrix	HSCR-SM
Human Soluble Cytokine Receptor Detection Antibodies	HSCRMG-1032
Streptavidin-Phycoerythrin	L-SAPE3
Assay Buffer	L-AB
Bead Diluent	LBD
Set of two 96-Well plates with sealers	MAG-PLATE
10X Wash Buffer	L-WB
Human Soluble Cytokine Receptor 14 Plex Premixed Magnetic Bead Panel – BULK PACKAGING	HSCRMAG32PMX14BK

Antibody-Immobilized Magnetic Beads

Analyte	*Bead #*	*Cat #*
Anti – sCD30 Bead	12	HCD30-MAG
Anti-sEGFR Bead	14	HEGFR-MAG
Anti – sgp130 Bead	18	HGP130-MAG
Anti – sIL-1RI Bead	20	HIL1R1-MAG
Anti – sIL-1RII Bead	22	HIL1R2-MAG
Anti – sIL-2Rα Bead	33	HSIL2RA-MAG
Anti – sIL-4R Bead	35	HIL4R-MAG
Anti – sIL-6R Bead	37	HIL6R-MAG
Anti – sRAGE Bead	39	HSRAGE-MAG
Anti – sTNFRI Bead	51	HTNFR1-MAG
Anti – sTNFRII Bead	53	HTNFR2-MAG
Anti – sVEGFR1 Bead	55	HVEGFR1-MAG
Anti – sVEGFR2 Bead	57	HVEGFR2-MAG
Anti – sVEGFR3 Bead	61	HVEGFR3-MAG
Premixed 13-plex Beads + HEGFR-MAG		HSCRPMX13-MAG + HEGFR-MAG

ORDERING INFORMATION

To place an order:

To assure the clarity of your custom kit order, please FAX the following information to our customer service department:

Include:

- Your name, telephone and/or fax number
- Customer account number
- Shipping and billing address
- Purchase order number
- Catalog number and description of product
- Quantity of kits
- Selection of MILLIPLEX® Analytes

FAX: (636) 441-8050

Toll-Free US: (866) 441-8400
(636) 441-8400

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

For European Customers:

To best serve our European customers in placing an order or obtaining additional information about MILLIPLEX® MAP products, please contact your multiplex specialist or sales representative or email our European Customer Service at:

Austria	AUCustomerService@merckgroup.com
Belgium	BENLCustomerService@merckgroup.com
Denmark	denmark@merckgroup.com
France	FRCustomerService@merckgroup.com
Finland	Asiakaspalvelu@merckgroup.com
Germany	GECustomerService@merckgroup.com
Ireland	IECustomerService@merckgroup.com
Italy	CSR-IT@merckgroup.com
Netherlands	BENLCustomerService@merckgroup.com
Norway	Norway@merckgroup.com
Spain	pedidos@merckgroup.com
Sweden	Kundservice@merckgroup.com
Switzerland	SZCustomerService@merckgroup.com
UK	UKCustomerService@merckgroup.com

ORDERING INFORMATION (continued)

Conditions of Sale

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Material Safety Data Sheets (MSDS)

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

Technical Services

http://www.emdmillipore.com/techservices

To contact by phone

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

Outside North America, contact your local office
http://www.emdmillipore.com/offices

WELL MAP

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

Human Pituitary Magnetic Bead Panel 1

96-Well Plate Assay

HPTP1MAG-66K

MILLIPLEX® MAP

Human Pituitary Magnetic Bead Panel 1
96-Well Plate Assay

HPTP1MAG-66K

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Human Pituitary Magnetic Bead Panel 1

INTRODUCTION

Pituitary hormones and other brain-derived poteins, such as hypothalamus neuropeptides, play very important roles in the regulation of various functions including metabolism, growth, and reproduction. Accurate measurement of these proteins to understand their new biological functions and molecular mechanisms of the functions are crucial. Traditional laboratory methods, such as RIA and ELISA are not able to measure multiple proteins with a small sample volume.
The MILLIPLEX® MAP Human Pituitary Magnetic Bead Panel 1 provides biomedical researchers with quality tools for the study of reproduction, growth, metabolic homeostasis, and pituitary-related diseases such as acromegaly, growth hormone deficiency, diabetes insipidus and pituitary tumors. MILLIPLEX® MAP enables you to investigate the modulation and expression of multiple analytes simultaneously, giving you the advantage of speed and sensitivity, and dramatically improving productivity.

MILLIPLEX® MAP offers the broadest selection of analytes across a wide range of disease states and species. Once the analytes of interest have been identified, you can rely on the quality that we build into each kit to produce results you can trust. In addition to the assay characteristics listed in the protocol, other performance criteria evaluated during the validation process include: cross-reactivity, dilution linearity, kit stability, and sample behavior (e.g. detectability and stability).

Each MILLIPLEX® MAP panel and kit includes:

- Quality controls (QCs) provided to qualify assay performance
- Comparison of standard (calibrator) and QC lots to a reference lot to ensure lot-to-lot consistency
- Optimized serum matrix to mimic native analyte environment
- Detection antibody cocktails designed to yield consistent analyte profiles within panel

In addition each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The **MILLIPLEX® MAP** Human Pituitary Magnetic Bead Panel 1 thus enables you to focus on the therapeutic potential of pituitary-related diseases. 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® MAP Human Pituitary Magnetic Bead Panel 1 is part of the most versatile system available for human pituitary hormone research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically validate and build the most comprehensive library available for protein detection and quantitation.

- MILLIPLEX® MAP offers you:
    - The ability to choose any combination of analytes from our panel of 7 analytes to design a custom kit that better meets your needs.
    - A convenient "all-in-one" box format that gives you the assurance that you will have all the necessary reagents you need to run your assay.

EMD Millipore's MILLIPLEX® MAP Human Pituitary Magnetic Bead Panel 1 is a 7-plex kit to be used for the simultaneous quantification of any or all of the following analytes in human serum, plasma, cerebrospinal fluid (CSF), tissue/cell lysate, and culture supernatant samples: ACTH, AGRP, CNTF, FSH, GH, LH, and TSH. This multiplex assay can analyze these 7 proteins simultaneously and uses a small sample volume 25 µL.

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

Please read entire protocol before use.

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

PRINCIPLE

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

- Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500 5.6 µm polystyrene microspheres or 80 6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- EMD Millipore provides three Luminex instruments to acquire and analyze data using two detection methods:
    - The Luminex analyzers Luminex 200™ and FLEXMAP 3D®, flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
    - The Luminex analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. EMD Millipore combines the streamlined data acquisition power of Luminex xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex instruments.

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

STORAGE CONDITIONS UPON RECEIPT

- Recommended storage for kit components is 2 - 8°C.
- For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze/thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibody, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8 °C

Reagents Supplied	Catalog Number	Volume	Quantity
Pituitary Standard	PIT-8046	lyophilized	1 vial
Pituitary Quality Controls 1 and 2	PIT-6046	lyophilized	2 vials
Set of one 96-Well black Plate with 2 sealers	----------	----------	1 plate 2 sealers
Assay Buffer	L-AB	30 mL	1 bottle
Serum Matrix **Note: Contains 0.08% Sodium Azide**	LHPT-SM	lyophilized	1 bottle
Bead Diluent	LBD	3.5 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	60 mL	1 bottle
Pituitary Detection Antibodies	PIT-1046	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE7	5.5 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

Human Pituitary Antibody Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable 7 Analytes (20X concentration, 200 µL)	
		Available	Cat. #
ACTH	12	✓	HACTH-MAG
AGRP	15	✓	HAGRP-MAG
CNTF	20	✓	HCNTF-MAG
FSH	26	✓	RFSH-MAG
GH	29	✓	HGH-MAG
LH	33	✓	HLH-MAG
TSH	61	✓	HTSH-MAG

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (EMD Millipore Catalog #SHEATHFLUID) or Luminex Drive Fluid (EMD Millipore 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. Aluminum Foil
6. Absorbent Pads
7. Rubber Bands
8. Laboratory Vortex Mixer
9. Sonicator (Branson Ultrasonic Cleaner Model # B200 or equivalent)
10. Titer Plate Shaker (Lab-Line Instruments, Model #4625, or equivalent)
11. Luminex® 200™, HTS, FLEXMAP 3D®, or MAGPIX® with xPONENT® software by Luminex® Corporation
12. Automatic Plate Washer for magnetic beads (BioTek® 405 LS and 405 TS, EMD Millipore Catalog #40-094, #40-095, #40-096, #40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog #40-285 or equivalent).

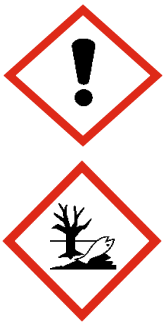
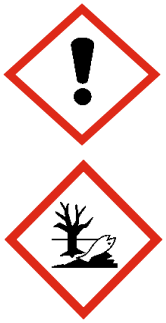
Note: If a plate washer or handheld magnetic separation block for magnetic beads is not available, one can use a microtiter filter plate (EMD Millipore Catalog #MX-PLATE) to run the assay using a Vacuum Filtration Unit (EMD Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with EMD Millipore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

SAFETY PRECAUTIONS

- All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.
- Sodium Azide or Proclin has been added to some reagents as a preservative. Although the concentrations are low, Sodium Azide and Proclin may react with lead and copper plumbing to form highly explosive metal azides. Dispose of unused contents and waste in accordance with international, federal, state, and local regulations.

Note: See Full Labels of Hazardous components on next page.

Full Labels of Hazardous components

Ingredient, Cat #		Full Label	
Serum Matrix	LHPT-SM		**Warning.** Harmful if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment.
Streptavidin-Phycoerythrin	L-SAPE7		**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.
Pituitary Detection Antibodies	PIT-1046		**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.
Pituitary Quality Controls 1 & 2	PIT-6046		**Warning.** Harmful if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment.
Pituitary Standard	PIT-8046		**Warning.** Harmful if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment.

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, dilute samples with Assay Buffer 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.
- 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 discsFor cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in blank, standard curve and controls.
- For serum / plasma samples, use the matrix provided in the kit in the kit as the matrix solution in blank, standard curve and controls.
- 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.

B. Preparation of Plasma Samples:

- Plasma collection using EDTA as an anticoagulant 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. If measuring ACTH, store plasma samples at -70°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.

C. Preparation of Tissue Culture Supernatant:

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

Note:

- 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

Sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 150 µL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Bead Diluent. Vortex the mixed beads well. Unused portions 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 7 antibody-immobilized beads, add 150 µL from each of the 7 bead sets to the Mixing Bottle. Then add 1.95 mL Bead Diluent.

Example 2: When using 3 antibody-immobilized beads, add 150 µL from each of the 3 bead sets to the Mixing Bottle. Then add 2.55 mL Bead Diluent.

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. Unused portions 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 with 540 mL deionized water. Store unused portions 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 1.0 mL Deionoized Water to the bottle containing lyophilized Serum Matrix. Allow at least 10 minutes for complete reconstitution. After reconstituition, add 1.0 mL of Assay Buffer to the bottle. Mix well. Leftover reconstituted Serum Matrix should be stored at ≤ -20°C for up to one month.

E. Preparation of Human Pituitary Standard

1.) Prior to use, reconstitute the Human Pituitary Standard with 250 µL Deionized Water. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. This will be used as Standard 7.

2.) Preparation of Working Standards

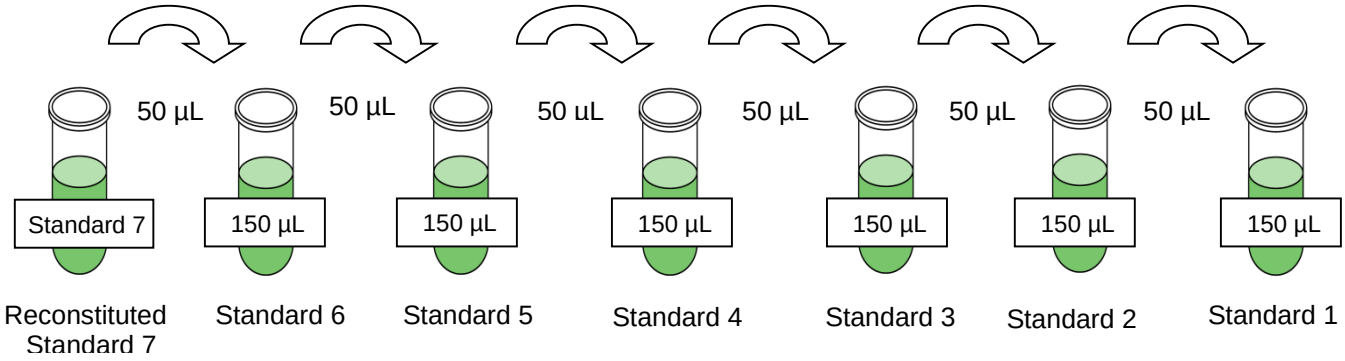
Label 6 polypropylene microfuge tubes “Standard 6,” “Standard 5,” “Standard 4,” “Standard 3,” “Standard 2,” and “Standard 1.” Add 150 µL Assay Buffer to each of the six tubes. Perform 4 times serial dilutions by adding 50 µL of the “Standard 7” to the “Standard 6” tube, mix well and transfer 50 µL of the “Standard 6” to the “Standard 5” tube, mix well and transfer 50 µL of the “Standard 5” to “Standard 4” tube, mix well and transfer 50 µL of the “Standard 4” to the “Standard 3”, 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”. The 0 Standard (background) will be Assay Buffer.

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

Reconstituted Standard	Volume of Deionized Water to Add	Volume of Standard to Add
Standard 7	250 µL	0

Standard	Volume of Assay Buffer to Add	Volume of Standard to Add
Standard 6	150 µL	50 µL of Standard 7
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 Working Standards



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

Standard Tube #	AGRP (pg/mL)	FSH (mIU/mL)	GH (pg/mL)	LH (mIU/mL)	TSH (µIU/mL)	ACTH (pg/mL)	CNTF (pg/mL)
1	2.4	0.024	2.4	0.049	0.039	3	122
2	10	0.098	10	0.195	0.156	12	488
3	39	0.39	39	0.781	0.625	49	1,953
4	156	1.56	156	3.125	2.5	195	7,813
5	625	6.25	625	12.5	10	781,	31,250
6	2,500	25	2,500	50	40	3,125	125,000
7	10,000	100	10,000	200	160	12,500	500,000

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), Std 1, Std 2, Std 3, Std 4, Std 5, Std 6, and Std 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 on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Add 200 µL of Assay Buffer into each well of the plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).
2. Decant Assay Buffer and remove the residual amount from all wells by inverting the plate and tapping it smartly onto absorbent towels several times.
3. Add 25 µL of Matrix Solution (when measuring serum or plasma samples) or appropriate culture media (when measuring culture samples) in Background, Standards, and Quality Control wells.
4. Add 25 µL Assay Buffer in Sample wells
5. Add 25 µL of Assay Buffer to the Background wells. Add 25 µL of each Standard or Control into the appropriate wells.
6. Add 25 µL of samples to the Sample wells.
7. Vortex Bead Bottle and add 25 µL of the prepared Beads to each well. (Note: during addition of the Beads, shake beads intermittently to avoid settling)
8. Seal the plate with a plate sealer (wrap the plate with foil if not using foil pate sealer) and incubate with agitation on a plate shaker for overnight incubation at 4°C *(16-18 hr).*

Add 200 µL Assay Buffer per well

↓ Shake 10 min, RT

↓ Decant

- Add 25 µL Matrix Solution (or appropriate media) to Background, Standard and Quality Controls.
- Add 25 µL Assay Buffer to Sample wells.
- Add 25 µL Assay Buffer to background wells and 25 µL Standard, and 25 µL Controls to Standard and Control wells, respectively.
- Add 25 µL samples to Sample Wells.

Add 25 µL Beads to each well

↓ Incubate overnight at 4°C with agitation on a plate shaker

9. Gently remove well contents and wash plate 2 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 60 minutes 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 100 µL of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 5 minutes.
16. Run plate on Luminex® 200™, HTS, FLEXMAP 3D® or MAGPIX® with xPONENT® software.
17. Save and analyze the median Fluorescent Intensity (MFI) data using a weighted 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples.

Remove well contents. Wash 2X with 200 µL Wash Buffer

Add 25 µL Detection Antibody per well

Incubate 60 min at RT

Do Not Aspirate

Add 25 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minute at RT

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

Add 100 µL Sheath Fluid or Drive Fluid per well

Read on Luminex (50 µL, 50 Beads per Bead set)

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PLATE WASHING

1.) Solid Plate

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

A.) Handheld magnet **(EMD Millipore Catalog #40-285)** - Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 µL of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

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

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

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 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

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

These specifications are for the Luminex 200™, Luminex HTS, Luminex FLEXMAP 3D®, and Luminex MAGPIX® with xPONENT® software. Luminex instruments with other software (e.g. MasterPlex®, STarStation, LiquiChip, Bio-Plex Manager™, LABScan™ 100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex magnetic beads.

For magnetic bead assays, the Luminex 200™ and HTS instruments must be calibrated with the xPONENT® 3.1 compatible Calibration Kit (EMD Millipore Catalog #40-275) and performance verified with the Performance Verification Kit (EMD Millipore Catalog #40-276). The Luminex FLEXMAP 3D® instrument must be calibrated with the FLEXMAP 3D® Calibrator Kit (EMD Millipore Catalog #40-028) and performance verified with the FLEXMAP 3D® Performance Verification Kit (EMD Millipore Catalog #40-029). The Luminex MAGPIX® instrument must be calibrated with the MAGPIX® Calibration Kit (EMD Millipore Catalog #40-049) and performance verified with the MAGPIX® Performance Verification Kit (EMD Millipore Catalog #40-050).

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

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

EQUIPMENT SETTINGS (continued)

The Luminex® probe height must be adjusted to the plate provided in the kit. Please use Cat# MAG-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 7-Plex Beads	
	ACTH Beads	12
	AGRP Beads	15
	CNTF Bead	20
	FSH Bead	26
	GH Bead	29
	LH Bead	33
	TSH Bead	61

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.emdmillipore.com using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Assay Sensitivities (minimum detectable concentrations)

Minimum Detectable Concentration (MinDC) is calculated using MILLIPLEX® Analyst 5.1. 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 = 6 assays)	
	Mean MinDC	Mean MinDc + 2SD
ACTH (pg/mL)	0.91	2.4
AGRP (pg/mL)	0.62	1.12
CNTF (pg/mL)	35.33	84.42
FSH (mIU/mL)	0.01	0.02
GH (pg/mL)	3.26	6.88
LH (mIU/mL)	0.01	0.02
TSH (µIU/mL)	0.01	0.02

Precision

Intra-assay precision is generated from the mean of the %CV's from 16 reportable results across three different concentrations of analytes in a single assay. Inter-assay precision is generated from the mean of the %CV's across eight different concentrations of analytes across six different assays.

Analyte	Intra-Assay CV %	Inter-Assay CV %
ACTH	< 10	< 15
AGRP	< 10	< 15
CNTF	< 10	< 15
FSH	< 10	< 15
GH	< 10	< 15
LH	< 10	< 15
TSH	< 10	< 15

ASSAY CHARACTERISTICS (continued)

Accuracy

Spike Recovery: The data represent mean percent recovery of spiked standards ranging from low, medium, and high concentration in serum samples (n=5).

Analyte	Spike and Recovery %
ACTH	100
AGRP	77
CNTF	84
FSH	97
GH	94
LH	87
TSH	105

Cross-Reactivity

The antibody pairs in the panel are specific only to the desired analyte and exhibit no or negligible cross-reactivity with other analytes in the panel.

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 to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height to the kit solid plate using 1 alignment disc. For FLEXMAP 3D® when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated.
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 Streptavidin-Phycoerythrin was not added	Add appropriate Detection Antibody and continue. 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 Incubations done at inappropriate temperatures, timings or agitation	May need to repeat assay if desired sensitivity not achieved. Assay conditions need to be checked.
Signals too high, standard curves are saturated	Calibration target value set too high Plate incubation was too long with standard curve and samples	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. Use shorter incubation time.
Sample readings are out of range	Samples contain no or below detectable levels of analyte Samples contain analyte concentrations higher than highest standard point. Standard curve was saturated at higher end of curve.	If below detectable levels, it may be possible to use higher sample volume. Check with tech support for appropriate protocol modifications. Samples may require dilution and reanalysis for just that particular analyte. See above.
High Variation in samples and/or standards	Multichannel pipet may not be calibrated Plate washing was not uniform Samples may have high particulate matter or other interfering substances Plate agitation was insufficient Cross well contamination	Calibrate pipets. Confirm all reagents are removed completely in all wash steps. See above. 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. 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		
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 setup and use supernatant.
	High lipid concentration	After centrifugation, remove lipid layer and use supernatant.
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 (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	**Cat #**
Pituitary Standard	PIT-8046
Pituitary Quality Controls	PIT-6046
Pituitary Detection Antibodies	PIT-1046
Serum Matrix	LHPT-SM
Bead Diluent	LBD
Assay Buffer	L-AB
Streptavidin-Phycoerythrin	L-SAPE7
Set of two 96-Well Black plates with 4 sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Beads

Analyte	*Bead #*	*Cat. #*
ACTH	12	HACTH-MAG
AGRP	15	HAGRP-MAG
CNTF	20	HCNTF-MAG
FSH	26	RFSH-MAG
GH	29	HGH-MAG
LH	33	HLH-MAG
TSH	61	HTSH-MAG

ORDERING INFORMATION

To place an order:

To assure the clarity of your custom kit order, please FAX the following information to our customer service department:

Include:

- Your name, telephone and/or fax number
- Customer account number
- Shipping and billing address
- Purchase order number
- Catalog number and description of product
- Quantity of kits
- Selection of MILLIPLEX® Analytes

FAX: (636) 441-8050

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ORDERING INFORMATION (continued)

Conditions of Sale

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

Material Safety Data Sheets (MSDS)

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

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

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http://www.emdmillipore.com/offices

WELL MAP

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

Magnetic Luminex® Performance Assay

Human MMP Base Kit

Catalog Number LMPM000

For the simultaneous quantitative determination of multiple human matrix metalloproteinase (MMP) concentrations in cell culture supernates, serum, plasma, platelet-poor plasma, saliva, and urine.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

The matrix metalloproteinases (MMPs) consist of 24 known human zinc proteases with essential roles in breaking down components of the extracellular matrix (ECM) (1-5). In addition to ECM proteins, other potential MMP substrates include cytokines (6-10), chemokines (11), growth factors and binding proteins (12-15), cell/cell adhesion molecules (16), and other proteinases (17, 18). With a few exceptions, MMPs share common structural motifs including a pro-peptide domain, a catalytic domain, a hinge region, and a hemopexin-like domain (2, 4, 5). Synthesized as pro-enzymes, most are secreted before conversion to their active forms. In general, the activation mechanism is thought to occur in a stepwise fashion involving disruption of the interaction between the catalytic site zinc and a cysteine-thiol group in the pro-peptide domain. This is followed by cleavage of the pro-peptide (5). Activation can be mediated by several serine proteases (19-21), MMPs (4, 17, 21-22), or potentially via NO-mediated S-nitrosylation of the pro-peptide cysteine-thiol group (23). In some cases, activation can take place intracellularly via a furin-like serine protease (24, 25). MMPs are expressed by many cell types and can be upregulated in response to adhesion molecules, growth factors, cytokines, and hormones (2-5). They have been implicated in several physiological processes including tissue morphogenesis (26-28), cell migration (29-31), wound healing (32), bone remodeling (33, 34), and angiogenesis (35-37). MMP activities are modulated on several levels including transcription, pro-enzyme activation, or by their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (5, 38). Imbalances in MMP regulation have been implicated in several pathological processes including cancer (39, 40), cardiovascular disorders (41, 42), and arthritis (43-45).

MMPs included in this panel:

Analyte	Catalog Number	Microparticle Region
EMMPRIN/CD147	LMPM972	30
MMP-1	LMPM901	20
MMP-2	LMPM902	19
MMP-3	LMPM513	21
MMP-7	LMPM907	22

Analyte	Catalog Number	Microparticle Region
MMP-8	LMPM908	25
MMP-9	LMPM911	26
MMP-10	LMPM910	27
MMP-12	LMPM919	28
MMP-13	LMPM511	29

PRINCIPLE OF THE ASSAY

Magnetic Luminex® Performance Assay multiplex kits are designed for use with the Luminex MAGPIX® CCD Imager. Alternatively, kits can be used with the Luminex 100/200™ or Bio-Rad® Bio-Plex®, dual laser, flow-based sorting and detection platforms.

Analyte-specific antibodies are pre-coated onto color-coded magnetic microparticles. Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the biotinylated antibody, is added to each well. A final wash removes unbound Streptavidin-PE, the microparticles are resuspended in buffer and read using the Luminex MAGPIX Analyzer. A magnet in the analyzer captures and holds the superparamagnetic microparticles in a monolayer. Two spectrally distinct Light Emitting Diodes (LEDs) illuminate the beads. One LED identifies the analyte that is being detected and the second LED determines the magnitude of the PE-derived signal, which is in direct proportion to the amount of analyte bound. Each well is imaged with a CCD camera. Kits can also be used with Luminex 100/200 or Bio-Rad Bio-Plex dual laser, flow-based systems.

LIMITATIONS OF THE PROCEDURE

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

TECHNICAL HINTS

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

PRECAUTIONS

MMPs are detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past the kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED, DILUTED, OR RECONSTITUTED MATERIAL
MMP Panel Standard Cocktail	894339	2 vials of recombinant human MMPs in a buffered protein base with preservatives; lyophilized.	Discard after use. Use a fresh standard for each assay.
Microparticle Diluent 3	895857	6 mL of a buffered protein base with preservatives.	May be stored for up to 1 month at 2-8 °C.* *Once diluted, any unused microparticle cocktail must be discarded.*
Biotin Antibody Diluent 2	895832	5.5 mL of a buffered protein base with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Calibrator Diluent RD5-37	895853	21 mL of a buffered protein base with preservatives.	
Streptavidin-PE	892525	0.07 mL of a 100-fold concentrated streptavidin-phycoerythrin conjugate with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Microplate	641385	1 flat-bottomed 96-well microplate used as a vessel for the assay.	
Mixing Bottles	895505	2 empty 8 mL bottles used for mixing microparticles with Microparticle Diluent.	
Plate Sealers	640445	4 adhesive foil strips.	
Standard Value Card	749407	1 card listing the Standard reconstitution volume and working standard concentrations for this lot of base kit.	

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

OTHER SUPPLIES REQUIRED

- **Luminex Performance Assay analyte-specific kit(s) (see Introduction on page 1).**
- Luminex MAGPIX, Luminex 100/200, or Bio-Rad Bio-Plex analyzer with X-Y platform.
- Hand-held microplate magnet or platewasher with a magnetic platform.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Multi-channel pipette, manifold dispenser, or automated dispensing unit.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 800 ± 50 rpm.
- Microcentrifuge.
- **Polypropylene** test tubes for dilution of standards and samples.

SAMPLE COLLECTION AND STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Cell culture supernate samples are not suitable for use in the MMP-2 assay.*

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifuging for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Platelet-poor Plasma* - Collect plasma on ice using heparin as an anticoagulant. Centrifuge at 2-8 °C for 15 minutes at 1000 x g within 30 minutes of collection. For complete platelet removal, centrifuge the separated plasma at 10,000 x g for 10 minutes at 2-8 °C. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Plasma and platelet-poor plasma samples are not suitable for use in the MMP-13 assay. EDTA and Citrate are not recommended for use in this assay due to their chelating properties. Hemolyzed and icteric samples are not suitable for use in this assay.*

***Some MMPs may be released upon platelet activation. For example, to measure circulating levels of MMP-9, platelet-poor plasma should be used. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical and Laboratory Standards Institute (CLSI), result in incomplete removal of platelets or platelet activation. This may cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation.**

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Use polypropylene tubes.

Note: *When assaying serum and plasma samples, EMMPRIN cannot be multiplexed with MMP-7, MMP-8, MMP-10, MMP-12, or MMP-13 (R&D Systems Catalog #'s LMPM907, LMPM908, LMPM910, LMPM919, and LMPM511, respectively).*

Cell culture supernate, serum, plasma, and platelet-poor plasma samples require a 5-fold dilution. A suggested 5-fold dilution is 40 µL of sample + 160 µL of Calibrator Diluent RD5-37. Mix thoroughly.

MMP-2, MMP-8, MMP-9, and MMP-12 serum and plasma samples must be further diluted 10-fold to a final 50-fold dilution. A suggested 50-fold dilution is 20 µL of the 5-fold diluted sample + 180 µL of Calibrator Diluent RD5-37. Mix thoroughly.

Saliva samples require a 40-fold dilution. A suggested 40-fold dilution is 10 µL of sample + 390 µL of Calibrator Diluent RD5-37. Mix thoroughly.

Urine samples require a 10-fold dilution. A suggested 10-fold dilution is 15 µL of sample + 135 µL of Calibrator Diluent RD5-37. Mix thoroughly.

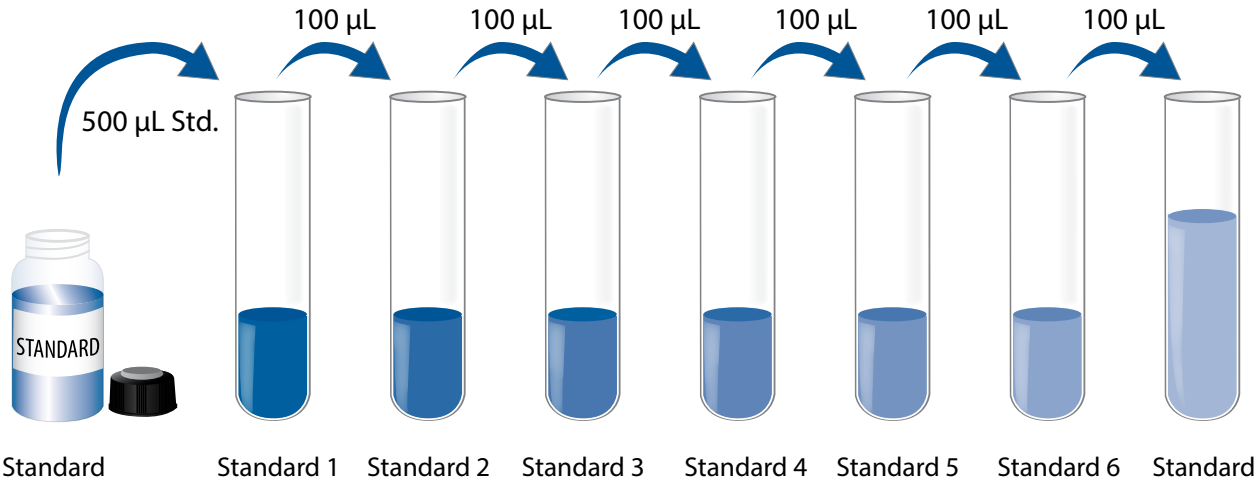
REAGENT PREPARATION

Bring all reagents to room temperature before use.

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

Standard - Reconstitute the Standard with Calibrator Diluent RD5-37. Refer to the Standard Value Card for the reconstitution volume and assigned values. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 500 µL of the reconstituted Standard into the Standard 1 tube. Pipette 200 µL of Calibrator Diluent RD5-37 into the remaining tubes. Use Standard 1 to produce a 3-fold dilution series (below). *See analyte specific datasheets for details.* Mix each tube thoroughly before the next transfer. Standard 1 serves as the high standard. Calibrator Diluent RD5-37 serves as the blank.



DILUTED MICROPARTICLE COCKTAIL PREPARATION

1. Centrifuge each Microparticle Concentrate vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vials to resuspend the microparticles, taking precautions not to invert the vials.
3. Dilute the Microparticle Concentrates in the mixing bottle provided. The volume of the Microparticle Concentrate listed in the table below is for each analyte (*e.g.* if measuring a full plate of MMP-1 and MMP-9, add 50 µL of MMP-1 Microparticle Concentrate and 50 µL of MMP-9 Microparticle Concentrate to 5 mL of Microparticle Diluent 3).

Number of Wells Used	Microparticle Concentrate	+	Microparticle Diluent 3
96	50.0 µL	+	5.00 mL
72	37.5 µL	+	3.75 mL
48	25.0 µL	+	2.50 mL
24	12.5 µL	+	1.25 mL

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

DILUTED BIOTIN ANTIBODY COCKTAIL PREPARATION

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

STREPTAVIDIN-PE PREPARATION

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

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

INSTRUMENT SETTINGS

Luminex MAGPIX analyzer:

a) Assign the microparticle region for each analyte being measured (see Introduction on page 1)

b) 50 events/bead

c) Sample size: 50 µL

d) Collect Median Fluorescence Intensity (MFI)

Luminex 100/200 and Bio-Rad Bio-Plex analyzers:

Note: *Calibrate the analyzer using the proper reagents for superparamagnetic microparticles (refer to instrument manual).*

a) Assign the bead region for each analyte being measured (see Introduction on page 1)

b) 50 events/bead

c) Minimum events: 0

d) Flow rate: 60 µL/minute (fast)

e) Sample size: 50 µL

f) Doublet Discriminator gates at approximately 8000 and 16,500

g) Collect MFI

Note: *The CAL2 setting for the Bio-Rad Bio-Plex analyzer should be set at the low RP1 target value.*

ASSAY PROCEDURE

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

Note: *Protect microparticles and Streptavidin-PE from light at all times.*
MMPs are detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Resuspend the diluted microparticle cocktail by inversion or vortexing. Add 50 µL of the microparticle cocktail to each well of the microplate.
3. Add 50 µL of Standard or sample* per well. Pipette assay within 15 minutes. Securely cover with a foil plate sealer. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 800 ± 50 rpm.
4. Using a magnetic device designed to accommodate a microplate, wash by applying the magnet to the bottom of the microplate, removing the liquid, filling each well with Wash Buffer (100 µL) and removing the liquid again. Complete removal of liquid is essential for good performance. Perform the wash procedure three times.

   Note: *Refer to the magnetic device user manual for proper wash technique using a round bottom microplate.*
5. Add 50 µL of diluted Biotin Antibody Cocktail to all wells. Securely cover with a foil plate sealer and incubate for 1 hour at room temperature on the shaker set at 800 ± 50 rpm.
6. Repeat the wash as in step 4.
7. Add 50 µL of diluted Streptavidin-PE to all wells. Securely cover with a foil plate sealer and incubate for 30 minutes at room temperature on the shaker set at 800 ± 50 rpm.
8. Repeat the wash as in step 4.
9. Resuspend the microparticles by adding 100 µL of Wash Buffer to each well. Incubate for 2 minutes on the shaker set at 800 ± 50 rpm.
10. Read within 90 minutes using the Luminex or Bio-Rad analyzer.
    Note: *Resuspend microparticles immediately prior to reading.*

*Samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Use the Standard concentrations on the Standard Value Card and calculate 3-fold dilutions for the remaining levels. Average the duplicate readings for each standard and sample and subtract the average blank Median Fluorescence Intensity (MFI).

Create a standard curve for each analyte by reducing the data using computer software capable of generating a five parameter logistic (5-PL) curve-fit.

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

CALIBRATION

This assay is calibrated against highly purified recombinant human MMPs produced at R&D Systems.

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IN-HOUSE Capture Sandwich Assay- Washed Protocol
I/2 Volumne with Overnight Incubation

- Pre-wet a filter plate using **75** ul of Wash Buffer (1X PBS+**1%** BSA+ 0.05% Tween) and seal the plate with a plastic cover.
- Place the filter plate on the inverted lid and position on the plate shaker till ready for use.
- To each bead stock bottle vortex for 30 sec, sonicate for 1 min, and again vortex for 30 sec.
- Prepare working microsphere mixture by diluting stock coupled microspheres to 2500 *of each analyte coupled microsphere bead/ul* with Wash Buffer (1X PBS+**1%** BSA+ 0.05% Tween)
- To the working bead mixture vortex for 30 sec, sonicate for 1 min, and again vortex for 30 sec.
- Prepare working standards in **SERUM SUBSTITUTE** (Scantibodies) by making a 10 point standard curve using serial dilutions at **1:2** in **Serum Substitute.** ***(The blank will only be*** **SERUM SUBSTITUTE**
- Ditue the samples **1:5** using (1X PBS+5% BSA+ 0.05% Tween) (requires 25ul/well)
  For example: 7ul of sample + 28ul (1X PBS+5% BSA+ 0.05% Tween) for a total of 35ul.
- Aspirate the sample plate on the vacuum apparatus before loading.
- Add **25**ul of **standard** or **sample** to the filter plate using a predetermined pattern.
- Add **25**ul of microsphere mixture to every well. Seal the plate with a plastic cover.
- Place the filter plate on the inverted lid, wrap the plate in foil (**protect from light**) and position on the plate shaker.
- Incubate overnight (16-18 hrs) at 4°C on a plate shaker set at speed 4.5.
- After the overnight incubation,allow the plate to come to room temperature.
- Wash the plate with **100** ul of Wash Buffer (1X PBS+**1%**BSA+0.05% Tween) and aspirate by vacuum. Repeat this step.
- Resuspend the microspheres in **25**ul of Wash Buffer(1X PBS+**1%**BSA+0.05% Tween).
- Dilute the biotinylated detection antibody in Wash Buffer (1X PBS+**1%**BSA+0.05% Tween). (requires 25ul/well) ***Concentrations of detection antibodies depend on the cytokine.***
- Add **25**ul of diluted detection antibody, Seal the plate with a plastic cover.
- Place the filter plate on the inverted lid, wrap the plate in foil (**protect from light**) and position on the plate shaker.
- Incubate for **2** hours at room temperature on a plate shaker set at speed 4.5. (**protect from light**)
- After the 2 hour incubation, wash the plate with**100** ul of Was Buffer (1X PBS+**1%**BSA+0.05% Tween) and aspirate by vacuum. Repeat this step.
- Resuspend the microspheres in **25**ul of Wash Buffer (1X PBS+**1%**BSA+0.05% Tween).
- Dilute the streptavidin-R-phycoerythrin to 4ug/ml in Wash Buffer (1X PBS+**1%**BSA+0.05% Tween). (requires 25ul/well)
- Add **25**ul of diluted streptavidin-PE. Seal the plate with a plastic cover.
- Place the filter plate on the inverted lid, wrap the plate in foil (**protect from light**) and position on the plate shaker..
- Incubate for **20** min at room temperature on a plate shaker set at speed 4.5. (**protect from light**)
- After the 20 min incubation, wash the plate with **100** ul of Wash Buffer (1X PBS+**1%**BSA+0.05% Tween) and aspirate by vacuum. Repeat this step.
- Resuspend the microspheres in 100ul of Wash Buffer (1X PBS+**1%**BSA+0.05% Tween). Seal the plate with a plastic cover.
- Place the filter plate on the inverted lid, wrap the plate in foil (**protect from light**) and position on the plate shaker for 5 min
- Analyze on the LUMINEX^{100}.
- Sample size 75 ul Bead events 100/bead