Mouse Angiogenesis /Growth Factor Magnetic Bead Panel

96-Well Plate Assay

Cat. # MAGPMAG-24K

MILLIPLEX® MAP

MOUSE ANGIOGENESIS / GROWTH FACTOR MAGNETIC BEAD PANEL

96-Well Plate Assay

MAGPMAG-24K

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INTRODUCTION

Angiogenesis, the development of new vascular networks, is a key process in normal growth and development as well as in wound healing, with homeostasis maintained by a delicate balance of angiogenic factors and inhibitors. Consequently, insufficient or excessive blood vessel growth underlies many diseases, including cardiovascular disease, diabetic ulcers, macular degeneration and cancer.

Angiogenesis plays a significant role in tumor growth and metastasis. In tumors, dysregulated signaling and hypoxic conditions lead to sustained, almost uncontrolled angiogenesis, a necessary component of tumor growth and metastasis. Chronic inflammation mechanisms, such as the production of reactive oxygen species during infection and secretion of proinflammatory cytokines, can also foster angiogenesis in tumor progression. Angiogenic signaling in tumors is similar to normal angiogenesis, mediated by soluble growth factors, membrane-bound receptors, and cell-cell and cell-matrix interactions. Such signaling regulates cell migration, which is vital to angiogenesis. However, there are multiple differences between tumor angiogenesis and normal blood vessel formation. Tumor endothelial cells proliferate faster than non-tumor endothelial cells. Tumor vasculature differs from normal vasculature in morphology, enhanced leakiness, and structural abnormalities. Finally, tumor vessels are often not capable of transporting oxygen to and removing waste products from all of the tumor tissues, resulting in frequent tumor cell necrosis.

To identify specific angiogenic processes, it might be necessary to screen panels of vascular analytes and growth factors, often requiring some level of automation and/or high throughput. Magnetic Beads can make the process of automation and high throughput screening easier with features such as walk-away washing. Advantages even outside automation include:

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

Therefore, the MILLIPLEX® MAP Mouse Angiogenesis / Growth Factor Magnetic Bead panel enables you to focus on your angiogenesis / vascular research. 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 Mouse Angiogenesis / Growth Factor Magnetic Bead panel is the most versatile system available for angiogenesis / vascular research.

- MILLIPLEX® MAP offers you the ability to:
    - Select a 24-plex (for serum/plasma) or 27-plex (for cell culture) or
    - Choose any combination of analytes from our panel of 27 analytes to design a custom kit that better meets your needs.
- A convenient "all-in-one" box format gives you the assurance that you will have all the necessary reagents you need to run your assay.

EMD Millipore's MILLIPLEX® MAP Mouse Angiogenesis / Growth Factor Magnetic Bead Panel is a 24-plex (for serum/plasma) or 27-plex (for cell culture) kit to be used for the simultaneous quantification of the following analytes: Angiopoietin-2, G-CSF, sFasL, sAlk-1, Amphiregulin, Leptin, IL-1β, Betacellulin, EGF, IL-6, Endoglin, Endothelin-1, FGF-2, Follistatin, HGF, PECAM-1, IL-17, PLGF-2, KC, MCP-1, Prolactin, MIP-1α, SDF-1, VEGF-C, VEGF-D, VEGF-A, and TNFα.

Note: This kit may be used for the analysis of all or any combination of the above analytes in cell culture supernatant samples. This kit can also be used in serum or plasma samples for the analysis of any combination of the above analytes except Angiopoietin-2, Betacellulin, and PECAM-1. These 3 analytes have not been analytically validated in serum/plasma samples.

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

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

STORAGE CONDITIONS UPON RECEIPT

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

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

Reagents Supplied	Catalog Number	Volume	Quantity
Mouse Angiogenesis Standard	MAGP-8024	Lyophilized	1 vial
Mouse Angiogenesis Quality Controls 1 and 2	MAGP-6024	Lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	MXMSM-2	Lyophilized	1 vial
Set of one 96-Well Plate with 2 sealers	----------	----------	1 plate 2 sealers
Assay Buffer	L-AB	30 mL	1 bottle
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	2 bottles
Mouse Angiogenesis Detection Antibodies	MAGP-1024	3.2 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE10	3.2 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

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

Mouse Angiogenesis / Growth Factor Antibody-Immobilized Magnetic Beads:

Bead/Analyte Name	Luminex Magnetic Bead Region	Customizable **27** Analytes (**50**X concentration, **90**µL) Available	Cat. #	**24**-Plex Beads **(serum/ plasma)**	**27**-Plex Beads **(cell culture)**
Anti-Angiopoietin-2 Bead	12	✓	MANGPT2-MAG		✓
Anti-G-CSF Bead	13	✓	MGCSF-MAG	✓	✓
Anti-sFasL Bead	14	✓	MFASL-MAG	✓	✓
Anti-sALK-1 Bead	15	✓	MSALK1-MAG	✓	✓
Anti-Amphiregulin Bead	18	✓	MAREG-MAG	✓	✓
Anti-Leptin Bead	22	✓	RCYLPTN-MAG	✓	✓
Anti-IL-1β Bead	25	✓	MIL1B-MAG	✓	✓
Anti-Betacellulin Bead	29	✓	MBTC-MAG		✓
Anti-EGF Bead	33	✓	MEGF-MAG	✓	✓
Anti-IL-6 Bead	34	✓	MCYIL6-MAG	✓	✓
Anti-Endoglin Bead	35	✓	MENDGLN-MAG	✓	✓
Anti-Endothelin-1 Bead	37	✓	MET1-MAG	✓	✓
Anti-FGF-2 Bead	39	✓	MFGF2-MAG	✓	✓
Anti-Follistatin Bead	42	✓	MFLSTN-MAG	✓	✓
Anti-HGF Bead	44	✓	MHGF-MAG	✓	✓
Anti-sCD31/PECAM-1 Bead	53	✓	MPCAM1-MAG		✓
Anti-IL-17A Bead	56	✓	MIL17-MAG	✓	✓
Anti-PLGF-2 Bead	57	✓	MPLGF2-MAG	✓	✓
Anti-KC Bead	61	✓	MKC-MAG	✓	✓
Anti-MCP-1 Bead	62	✓	MCYMCP1-MAG	✓	✓
Anti-Prolactin Bead	63	✓	MANGPRL-MAG	✓	✓
Anti-MIP-1α Bead	64	✓	MMIP1A-MAG	✓	✓
Anti-SDF-1 Bead	65	✓	MSDF1-MAG	✓	✓
Anti-VEGF-C Bead	72	✓	MVEGFC-MAG	✓	✓
Anti-VEGF-D Bead	74	✓	MVEGFD-MAG	✓	✓
Anti-VEGF-A Bead	76	✓	MVEGF-MAG	✓	✓
Anti-TNFα Bead	77	✓	MCYTNFA-MAG	✓	✓

Note: Angiopoeitin-2, Betacellulin, and PECAM-1 should not be run in serum/plasma samples.

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex® Sheath Fluid (Luminex Catalog #40-50000) or Luminex® Drive Fluid (Luminex Catalog #MPXDF-4PK)

Instrumentation / Materials

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

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.

TECHNICAL GUIDELINES (continued)

- 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 using 3 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 2 alignment discs.
- For FLEXMAP 3D® when using the solid plate in the kit, the final suspension should be in 150 µL and 75 µL should be aspirated.
- For cell culture supernatants, use the culture medium as the matrix solution in the background, standard curve and control wells. If samples are diluted in assay buffer, use the assay buffer as matrix.
- For serum/plasma samples that require further dilution beyond 1:2 dilution, 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. When use MILLIPLEX® MAP Lysis Buffer (43-040), dilute the clear lysates at least 1:4 in assay buffer, use the diluted lysis buffer as matrix solution.
- 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 in the Assay Buffer provided in the kit. For example, in a tube, 40 µL of serum may be combined with 40 µL of Assay Buffer. When further dilution beyond 1:2 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 1:2 in the Assay Buffer provided in the kit. For example, in a tube, 40 µL of plasma may be combined with 40 µL of Assay Buffer. When further dilution beyond 1:2 is required, use Serum Matrix as the diluent.

C. Preparation of Tissue Culture Supernatant and Tissue/Cell Extracts:

- 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.
- When using MILLIPLEX® MAP Lysis Buffer (43-040) in preparing cell/tissue lysates, it is recommended to dilute the clear lysates at least 1:4 in assay buffer provided in the kit. For example, in a tube, 20 µL of lysate may be combined with 60 µL of Assay Buffer. When 1:4 dilulted lysate is used, use 1:4 diluted lysis buffer as the matrix solution.

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 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

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 the provided Assay Buffer. Vortex the mixed beads well. Unused portion may be stored at 2-8°C for up to one month. (Note: Due to the composition of magnetic beads, you may notice a slight color in the bead solution. This does not affect the performance of the beads or the kit.)

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

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

B. Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 µL deionized water, respectively. 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 (two bottles) with 540 mL deionized water. Store unused portion at 2-8°C for up to one month.

D. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

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

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

E. Preparation of Mouse Angiogenesis Standard

1.) Prior to use, reconstitute the Mouse Angiogenesis 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 the Standard 7; the unused portion may be stored at ≤ -20°C for up to one month.

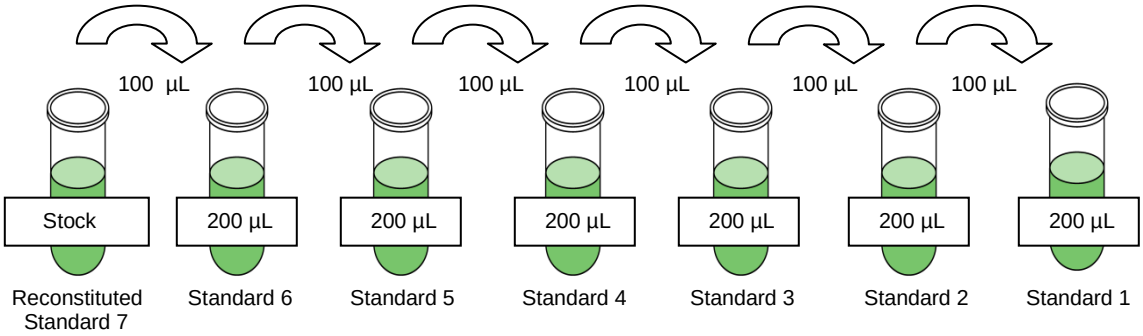
2). Preparation of Working Standards

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 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 standard (Background) will be Assay Buffer.

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

Standard Concentration (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

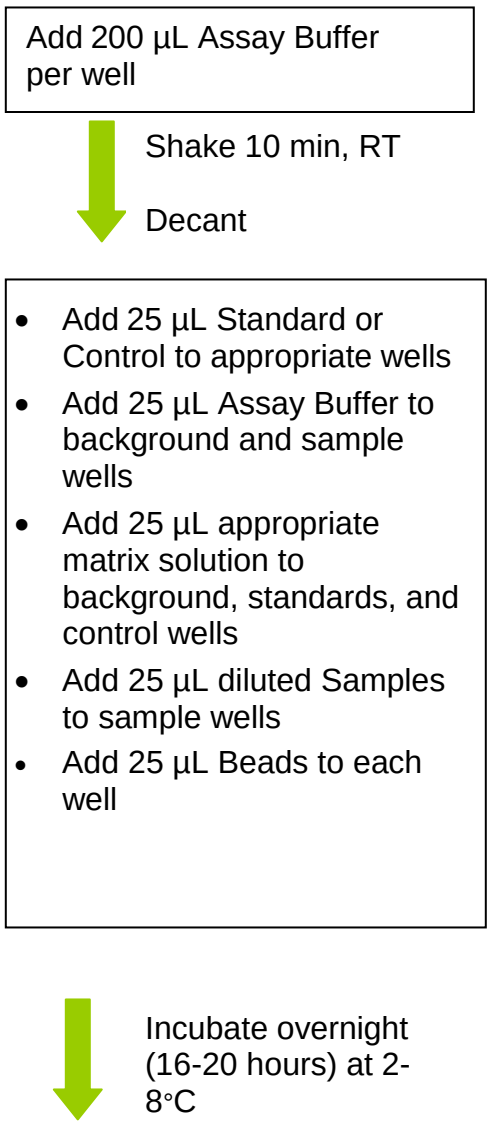


Standard	Endo-thelin-1 (pg/mL)	PLGF-2 (pg/mL)	VEGF-A, TNFα (pg/mL)	G-CSF, Amphiregulin, Betacellulin, IL-6, IL-17A, KC (pg/mL)	sALK-1, Leptin, IL-1β, PECAM-1, MIP-1α (pg/mL)	Angio-poietin-2, EGF, Endoglin, MCP-1, VEGF-C (pg/mL)	sFasL, FGF-2, Prolactin, VEGF-D (pg/mL)	Follistatin, HGF, SDF-1 (pg/mL)
Standard 1	1.4	2.7	5.5	6.9	13.7	27.4	68.6	137.2
Standard 2	4.1	8.2	16.5	20.6	41.2	82.3	205.8	411.5
Standard 3	12.3	24.7	49.4	61.7	123.5	246.9	617.3	1,234.6
Standard 4	37.0	74.1	148.1	185.2	370.4	740.7	1,851.9	3,703.7
Standard 5	111.1	222.2	444.4	555.6	1,111.1	2,222.2	5,555.6	11,111.1
Standard 6	333.3	666.7	1,333.3	1,666.7	3,333.3	6,666.7	16,666.7	33,333.3
Standard 7	1,000	2,000	4,000	5,000	10,000	20,000	50,000	100,000

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), Standard 1, 2, 3, 4, 5, 6 and 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. 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 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 for overnight (16-20 hours) at 2-8°C.



9. Remove solution in the wells by decanting/aspiration/vacuuming 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. Remove solution in the wells by decanting/aspiration/vacuuming and wash plate 3 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.)

Decant/Aspirate/ Vacuum 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

Decant/Aspirate/ Vacuum and wash 3X with 200 µL Wash Buffer

Add 150 µL Sheath Fluid or Drive Fluid per well

Read on Luminex® (100µL, 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.) For handheld magnet, rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Attach the plate to the Magnetic Separation Block and decant the solution in the wells into 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 the plate to magnet, letting beads settle for 60 seconds and decanting the solution in the wells as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.

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

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

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

EQUIPMENT SETTINGS

BioTek® ELx405:

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

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

1.) Soak program:

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

2.) Wash program:

Method:

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

EQUIPMENT SETTINGS (continued)

Dispense:

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

Aspiration:

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

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

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

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

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

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

Events:	50, per bead	
Sample Size:	100 µL	
Gate Settings:	8,000 to 15,000	
Reporter Gain:	Default (low PMT)	
Time Out:	60 seconds	
Bead Set:	Customizable 27-Plex Beads	
	Angiopoietin-2	12
	G-CSF	13
	sFasL	14
	sALK-1	15
	Amphiregulin	18
	Leptin	22
	IL-1β	25
	Betacellulin	29
	EGF	33
	IL-6	34
	Endoglin	35
	Endothelin-1	37
	FGF-2	39
	Follistatin	42
	HGF	44
	sCD31/PECAM-1	53
	IL-17A	56
	PLGF-2	57
	KC	61
	MCP-1	62
	Prolactin	63
	MIP-1α	64
	SDF-1	65
	VEGF-C	72
	VEGF-D	74
	VEGF-A	76
	TNFα	77

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 Corporation website www.millipore.com/techlibrary/index.do using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Cross-Reactivity

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

Assay Sensitivities (minimum detectable concentrations, pg/mL)

Minimum Detectable Concentration (MinDC) is calculated using MILLIPLEX® Analyst software. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions.

Analyte	Overnight Protocol (n = 8 Assays)	
	MinDC (pg/mL)	MinDC+2SD (pg/mL)
Angiopoietin-2	5.2	17.3
G-CSF	0.8	3.5
sFasL	6.3	24.8
sALK-1	3.8	13.0
Amphiregulin	2.2	7.3
Leptin	1.9	7.1
IL-1β	2.2	7.2
Betacellulin	2.2	6.7
EGF	4.5	19.7
IL-6	0.5	2.2
Endoglin	2.2	13.2
Endothelin-1	0.4	1.3
FGF-2	10.6	78.4
Follistatin	50.3	150.3
HGF	15.9	70.9
PECAM-1	1.0	6.2
IL-17A	0.6	2.7
PLGF-2	0.2	0.7
KC	1.0	3.6
MCP-1	4.0	15.4
Prolactin	7.0	28.6
MIP-1α	1.3	7.4
SDF-1	24.6	120.4
VEGF-C	5.2	18.5
VEGF-D	21.3	75.4
VEGF-A	0.7	3.2
TNFα	0.9	4.1

ASSAY CHARACTERISTICS (continued)

Precision

Intra-assay precision is generated from the mean of the %CV's from 12 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
Angiopoietin-2	2.1	4.5
G-CSF	2.8	4.4
sFasL	5.1	4.6
sALK-1	3.7	5.5
Amphiregulin	2.8	5.7
Leptin	4.0	5.0
IL-1β	2.7	3.2
Betacellulin	2.4	4.0
EGF	3.3	3.6
IL-6	2.9	5.1
Endoglin	3.8	4.4
Endothelin-1	2.5	4.0
FGF-2	9.1	11.5
Follistatin	2.2	4.3
HGF	4.0	6.5
PECAM-1	2.8	4.7
IL-17A	2.5	5.1
PLGF-2	2.9	4.5
KC	3.0	6.0
MCP-1	2.6	5.2
Prolactin	3.9	3.9
MIP-1α	2.1	4.1
SDF-1	5.4	7.5
VEGF-C	5.6	5.5
VEGF-D	6.8	6.3
VEGF-A	2.5	5.1
TNFα	2.6	5.6

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
Angiopoietin-2	99
G-CSF	96
sFasL	98
sALK-1	98
Amphiregulin	97
Leptin	99
IL-1β	99
Betacellulin	95
EGF	97
IL-6	97
Endoglin	100
Endothelin-1	104
FGF-2	100
Follistatin	98
HGF	100
PECAM-1	99
IL-17A	94
PLGF-2	102
KC	96
MCP-1	97
Prolactin	97
MIP-1α	99
SDF-1	90
VEGF-C	99
VEGF-D	102
VEGF-A	102
TNFα	98

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 according to the protocols recommended by Luminex to the kit solid plate using 3 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 2 alignment discs. For FLEXMAP 3D® when using the solid plate in the kit, the final suspension should be in 150 µL 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 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 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 #**
Mouse Angiogenesis Standard	MAGP-8024
Mouse Angiogenesis Quality Controls 1 & 2	MAGP-6024
Serum Matrix	MXMSM-2
Mouse Angiogenesis Panel Detection Antibodies	MAGP-1024
Streptavidin-Phycoerythrin	L-SAPE10
Assay Buffer	L-AB
Set of two 96-Well plates with sealers	MAG-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Magnetic Beads

Analyte	*Bead #*	*Cat. #*
Anti-Angiopoietin-2 Bead	12	MANGPT2-MAG
Anti-G-CSF Bead	13	MGCSF-MAG
Anti-sFasL Bead	14	MFASL-MAG
Anti-sALK-1 Bead	15	MSALK1-MAG
Anti-Amphiregulin Bead	18	MAREG-MAG
Anti-Leptin Bead	22	RCYLPTN-MAG
Anti-IL-1β Bead	25	MIL1B-MAG
Anti-Betacellulin Bead	29	MBTC-MAG
Anti-EGF Bead	33	MEGF-MAG
Anti-IL-6 Bead	34	MCYIL6-MAG
Anti-Endoglin Bead	35	MENDGLN-MAG
Anti-Endothelin-1 Bead	37	MET1-MAG
Anti-FGF-2 Bead	39	MFGF2-MAG
Anti-Follistatin Bead	42	MFLSTN-MAG
Anti-HGF Bead	44	MHGF-MAG
Anti-sCD31/PECAM-1 Bead	53	MPCAM1-MAG
Anti-IL-17A Bead	56	MIL17-MAG
Anti-PLGF-2 Bead	57	MPLGF2-MAG
Anti-KC Bead	61	MKC-MAG
Anti-MCP-1 Bead	62	MCYMCP1-MAG
Anti-Prolactin Bead	63	MANGPRL-MAG
Anti-MIP-1α Bead	64	MMIP1A-MAG
Anti-SDF-1 Bead	65	MSDF1-MAG
Anti-VEGF-C Bead	72	MVEGFC-MAG
Anti-VEGF-D Bead	74	MVEGFD-MAG
Anti-VEGF-A Bead	76	MVEGF-MAG
Anti-TNFα Bead	77	MCYTNFA-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 International Customers:

To best serve our international 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 customerserviceEU@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.millipore.com/techlibrary/index.do

Technical Services

http://www.millipore.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.millipore.com/offices

WELL MAP

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 Standard pg/mL (Background)	Standard 4	QC-1 Control	Etc.								
B	0 Standard pg/mL (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									