

User Guide

MILLIPLEX[®] 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.

The MILLIPLEX® portfolio 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 verification process include: cross-reactivity, dilution linearity, kit stability, and sample behavior (for example, detectability and stability).

Each MILLIPLEX® 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® Mouse Angiogenesis/Growth Factor Magnetic Bead Panel thus enables you to focus on the therapeutic potential of angiogenesis. 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.

The MILLIPLEX® Mouse Angiogenesis/Growth Factor Magnetic Bead Panel is part of the most versatile system available for angiogenesis/vascular research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically verify and build the most comprehensive library available for protein detection and quantitation.

MILLIPLEX® products offer you:

- The selection of a 24-plex (for serum/plasma) or 27-plex (for cell culture) or
- The ability to 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 that gives you the assurance that you will have all the necessary reagents you need to run your assay.

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

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.

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

- Luminex® products use proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets of 500-5.6 µm polystyrene microspheres or 80-6.45 µm magnetic microspheres can be created, each of which is coated with a specific capture antibody.
- After an analyte from a test sample is captured by the bead, a biotinylated detection antibody is introduced.
- The reaction mixture is then incubated with Streptavidin-PE conjugate, the reporter molecule, to complete the reaction on the surface of each microsphere.
- The following Luminex® instruments can be used to acquire and analyze data using two detection methods:
 - The Luminex® analyzers, Luminex® 200™, FLEXMAP 3D®, and xMAP® INTELLIFLEX, are flow cytometry-based instruments that integrate key xMAP® detection components, such as lasers, optics, advanced fluidics and high-speed digital signal processors.
 - The Luminex® analyzer (MAGPIX®), a CCD-based instrument that integrates key xMAP® capture and detection components with the speed and efficiency of magnetic beads.
- Each individual microsphere is identified and the result of its bioassay is quantified based on fluorescent reporter signals. We combine the streamlined data acquisition power of Luminex® xPONENT® acquisition software with sophisticated analysis capabilities of the new MILLIPLEX® Analyst 5.1, integrating data acquisition and analysis seamlessly with all Luminex® instruments.
- xMAP® INTELLIFLEX runs on INTELLIFLEX software for instrument control, run setup and generating high quality data with flexible output options. Data can be exported in xPONENT® style CSV files for compatibility with many existing analytical applications, or in the new, customizable INTELLIFLEX file format. The INTELLIFLEX file format is intended for flexibility and simplicity, allowing the user to freely select which data points to include and to reduce the time to analysis.

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

Storage Conditions Upon Receipt

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

Reagents Supplied

Store all reagents at 2-8 °C

Reagents	Volume	Quantity	Cat. No.
Mouse Angiogenesis Standard	Lyophilized	1 vial	MAGP-8024
Mouse Angiogenesis Quality Controls 1 and 2	Lyophilized	2 vials	MAGP-6024
Serum Matrix*	Lyophilized	1 vial	MXMSM-2
Set of one 96-Well Plate with 2 sealers	-	1 set	-
Assay Buffer	30 mL	1 bottle	L-AB
10X Wash Buffer**	60 mL	1 bottle	L-WB
Mouse Angiogenesis Detection Antibodies	3.2 mL	1 bottle	MAGP-1024
Streptavidin-Phycoerythrin	3.2 mL	1 bottle	L-SAPE10
Mixing Bottle	-	1 bottle	-

* Contains 0.08% Sodium azide

** Contains 0.05% Proclin

Included Mouse Angiogenesis/Growth Factor Antibody-Immobilized Magnetic Beads are dependent on customizable selection of analytes within the panel.

Mouse Angiogenesis/Growth Factor Antibody-Immobilized Magnetic Beads

Bead/Analyte Name	Luminex® Magnetic Bead Region	Customizable 27 Analytes (50X concentration, 90µL) Available	Cat. No.	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	✓	MCYTNTFA-MAG	✓	✓

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

Materials Required (not included)

Reagents

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

Instrumentation/Materials








- Adjustable pipettes with tips capable of delivering 25 µL to 1000 µL
- Multichannel pipettes capable of delivering 5 µL to 50 µL, or 25 µL to 200 µL
- Reagent reservoirs
- Polypropylene microfuge tubes
- Rubber bands
- Aluminum foil
- Absorbent pads
- Laboratory vortex mixer
- Sonicator (Branson Ultrasonic Cleaner Model B200 or equivalent)
- Titer plate shaker (VWR® Microplate Shaker Cat. No. 12620-926 or equivalent)
- Luminex® 200™, HTS, FLEXMAP 3D®, MAGPIX® instrument with xPONENT® software, or xMAP® INTELLIFLEX instrument with INTELLIFLEX software by Luminex® Corporation
- Automatic plate washer for magnetic beads (BioTek® 405 LS and 405 TS, Cat. No. 40-094, 40-095, 40-096, 40-097 or equivalent) or Handheld Magnetic Separation Block (Cat. No. 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 (Cat. No. MX-PLATE) to run the assay using a vacuum filtration unit (Vacuum Manifold, Cat. No. MSVMHTS00 or equivalent with Vacuum Pump, Cat. No. 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.

Symbol Definitions

Ingredient	Cat. No.	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.
10X Wash Buffer - MILLIPLEX	L-WB		Warning. May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.
Mouse Angiogenesis Detection Antibody	MAGP-1024		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.
Mouse Angiogenesis Quality Control 1,2	MAGP-6024	 	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.
Mouse Angiogenesis Standard	MAGP-8024	 	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	MXMSM-2	No Symbol Required	Harmful 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, 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 the Luminex® 200™ instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended filter plates using 3 alignment discs. When reading the assay on the MAGPIX® instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate or to the recommended filter plates using 2 alignment discs. When reading the assay on the FLEXMAP 3D® instrument, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 1 alignment disc.
- For the FLEXMAP 3D® instrument, when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid PLUS in each well and 75 µL should be aspirated.
- For the xMAP® INTELLIFLEX instrument, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.
- For cell culture supernatants, 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® 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

Preparation of Serum Samples

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C.
- Avoid multiple (> 2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Serum samples should be diluted 1:2 in the Assay Buffer provided in the kit. For example, in a tube, 40 μ L of serum may be combined with 40 μ L of Assay Buffer. When further dilution beyond 1:2 is required, use Serum Matrix as the diluent.

Preparation of Plasma Samples

- Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1000 x g within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at ≤ -20 °C.
- Avoid multiple (> 2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- Plasma samples should be diluted 1:2 in the Assay Buffer provided in the kit. For example, in a tube, 40 μ L of plasma may be combined with 40 μ L of Assay Buffer. When further dilution beyond 1:2 is required, use Serum Matrix as the diluent.

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® 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 diluted 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

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.

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. Transfer the reconstituted Quality Control 1 and Quality Control 2 into two polypropylene microfuge tubes. Unused portion may be stored at ≤ 20 °C for up to one month.

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.

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.

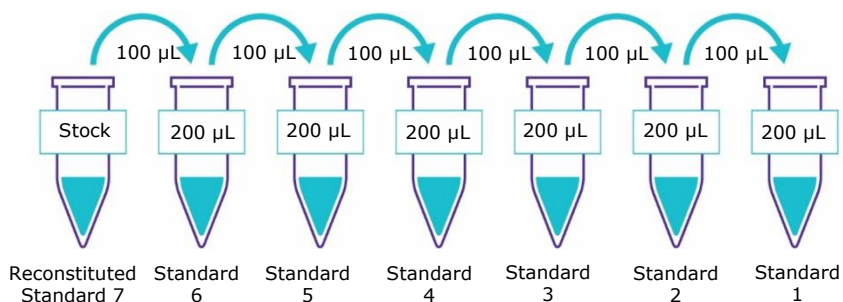
Preparation of Mouse Angiogenesis Standard

1. Prior to use, reconstitute the Mouse Angiogenesis Standard with 250 μ L 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. Transfer the reconstituted standard to a polypropylene microfuge tube. 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 μ L of Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 100 μ L of the reconstituted Standard 7 to the Standard 6 tube, mix well and transfer 100 μ L of the Standard 6 to the Standard 5 tube, mix well and transfer 100 μ L of the Standard 5 to the Standard 4 tube, mix well and transfer 100 μ L of the Standard 4 to Standard 3 tube, mix well and transfer 100 μ L of the Standard 3 to the Standard 2 tube, mix well and transfer 100 μ L of the Standard 2 to the Standard 1 tube and mix well. The 0 standard (Background) will be Assay Buffer.

Standard Tube No.	Add Deionized Water (μL)	Add Standard (volume)
Standard 7 (Reconstituted Standard)	250	0

Standard Tube No.	Add Assay Buffer (μL)	Add Standard (volume)
Standard 6	200	100 μL of Standard 7
Standard 5	200	100 μL of Standard 6
Standard 4	200	100 μL of Standard 5
Standard 3	200	100 μL of Standard 4
Standard 2	200	100 μL of Standard 3
Standard 1	200	100 μL of Standard 2

Preparation of Standards



Standard	Endothelin-1 (pg/mL)	PLGF-2 (pg/mL)	VEGF-A, TNFa (pg/mL)	G-CSF, Amphiregulin, Betacellulin, IL-6, IL-17A, KC (pg/mL)
Standard 1	1.4	2.7	5.5	6.9
Standard 2	4.1	8.2	16.5	20.6
Standard 3	12.3	24.7	49.4	61.7
Standard 4	37.0	74.1	148.1	185.2
Standard 5	111.1	222.2	444.4	555.6
Standard 6	333.3	666.7	1,333.3	1,666.7
Standard 7	1,000	2,000	4,000	5,000

Standard	sALK-1, Leptin, IL-1b, PECAM-1, MIP-1a (pg/mL)	Angiopoietin-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	13.7	27.4	68.6	137.2
Standard 2	41.2	82.3	205.8	411.5
Standard 3	123.5	246.9	617.3	1,234.6
Standard 4	370.4	740.7	1,851.9	3,703.7
Standard 5	1,111.1	2,222.2	5,555.6	11,111.1
Standard 6	3,333.3	6,666.7	16,666.7	33,333.3
Standard 7	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.

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



Incubate 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 $^{\circ}\text{C}$). **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 $^{\circ}\text{C}$).
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 PLUS (or Drive Fluid PLUS 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[®], MAGPIX[®] instrument with xPONENT[®] software or xMAP[®] INTELLIFLEX instrument with INTELLIFLEX 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
PLUS or Drive Fluid PLUS
per well

Read on Luminex[®]
instrument (100 μL ,
50 beads per bead set)

Plate Washing

Note: The use of a plate washer is not a recommended method of washing for MAGPMAG-24K assays. Deterioration of assay performance and well-to-well variability have been noted when using plate washers.

Solid Plate

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

- 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 μ L 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.
- 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 μ L/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**.

Filter Plate (Cat. No. MX-PLATE)

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 μ L/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®, MAGPIX® instruments with xPONENT® software and xMAP® INTELLIFLEX instrument with INTELLIFLEX software:

These specifications are for the above listed instruments and software. Luminex® instruments with other software (for example, MasterPlex®, StarStation, LiquiChip, Bio-Plex® Manager™, LABScan™100) would need to follow instrument instructions for gate settings and additional specifications from the vendors for reading Luminex® magnetic beads.

For magnetic bead assays, each instrument must be calibrated and performance verified with the indicated calibration and verification kits.

Instrument	Calibration Kit	Verification Kit
Luminex® 200™ and HTS	xPONENT® 3.1 compatible Calibration Kit (Cat. No. LX2R-CAL-K25)	Performance Verification Kit (Cat. No. LX2R-PVER-K25)
FLEXMAP 3D®	FLEXMAP 3D® Calibrator Kit (Cat. No. F3D-CAL-K25)	FLEXMAP 3D® Performance Verification Kit (Cat. No. F3D-PVER-K25)
xMAP® INTELLIFLEX	xMAP® INTELLIFLEX Calibration Kit (Cat. No. IFX-CAL-K20)	xMAP® INTELLIFLEX Performance Verification Kit (Cat. No. IFX-PVER-K20)
MAGPIX®	MAGPIX® Calibration Kit (Cat, No. MPX-CAL-K25)	MAGPIX® Performance Verification Kit (Cat. No. MPX-PVER-K25)

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.

The Luminex® probe height must be adjusted to the plate provided in the kit. Please use Cat. No. MAG-PLATE, if additional plates are required for this purpose.

Events	50, per bead
Sample Size	100 μ L
Gate Settings	8,000 to 15,000
Reporter Gain	Default (low PMT)
Time Out	60 seconds
Bead Set	Customizable 27-Plex Beads
	<hr/>
Angiopoietin-2	12
G-CSF	13
sFasL	14
	<hr/>
sALK-1	15
Amphiregulin	18
Leptin	22
	<hr/>
IL-1b	25
Betacellulin	29
EGF	33
	<hr/>
IL-6	34
Endoglin	35
Endothelin-1	37
	<hr/>
FGF-2	39
Follistatin	42
HGF	44
	<hr/>
sCD31/PECAM-1	53
IL-17A	56
PLGF-2	57
	<hr/>
KC	61
MCP-1	62
Prolactin	63
	<hr/>
MIP-1a	64
SDF-1	65
VEGF-C	72
	<hr/>
VEGF-D	74
VEGF-A	76
TNFa	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 our website [SigmaAldrich.com](https://www.sigmaaldrich.com) using the catalogue number as the keyword.

Assay Characteristics

Cross-Reactivity

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

Assay Sensitivities (minimum detectable concentrations, pg/mL)

Minimum Detectable Concentration (MinDC) is calculated using 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.

Overnight Protocol (n = 8 Assays)

Analyte	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-1b	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-1a	1.3	7.4
SDF-1	24.6	120.4
VEGF-C	24.6	52.9
VEGF-D	21.3	75.4
VEGF-A	0.7	3.2
TNFa	0.9	4.1

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.

Overnight Protocol

Analyte	Intra-assay %CV	Inter-assay %CV
Angiopoietin-2	< 10	< 10
G-CSF	< 10	< 10
sFasL	< 10	< 10
sALK-1	< 10	< 10
Amphiregulin	< 10	< 10
Leptin	< 10	< 10
IL-1b	< 10	< 10
Betacellulin	< 10	< 10
EGF	< 10	< 10
IL-6	< 10	< 10
Endoglin	< 10	< 10
Endothelin-1	< 10	< 10
FGF-2	< 10	< 15
Follistatin	< 10	<10
HGF	< 10	< 10
PECAM-1	< 10	< 10
IL-17A	< 10	< 10
PLGF-2	< 10	< 10
KC	< 10	< 10
MCP-1	< 10	< 10
Prolactin	< 10	< 10
MIP-1a	< 10	< 10
SDF-1	< 10	< 10
VEGF-C	< 10	< 10
VEGF-D	< 10	< 10
VEGF-A	< 10	< 10
TNFa	< 10	< 10

Accuracy

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

Overnight Protocol	
Analyte	% Recovery in Serum Matrix
Angiopoietin-2	99
G-CSF	96
sFasL	98
sALK-1	98
Amphiregulin	97
Leptin	99
IL-1b	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-1a	99
SDF-1	90
VEGF-C	87
VEGF-D	102
VEGF-A	102
TNFa	98

Troubleshooting

Problem	Probable Cause	Solution
	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.
Insufficient bead count	Probe height not adjusted correctly	When reading the assay on the Luminex® 200™ instrument, adjust probe height to the kit solid plate or to the recommended filter plates using 3 alignment discs. When reading the assay on MAGPIX®, adjust probe height to the kit solid plate or to the recommended filter plates using 2 alignment discs. When reading the assay on the FLEXMAP 3D® instrument, adjust probe height to the kit solid plate using 1 alignment disc. For the FLEXMAP 3D® instrument, when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid PLUS in each well and 75 µL should be aspirated. When reading the assay on the xMAP® INTELLIFLEX instrument, adjust probe height based on the type of plate you are using, place an alignment disk or an alignment sphere in the well according to the protocol recommended by Luminex®.
	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately and pipetting with multichannel pipettes without touching reagent in plate.
Background is too high	Matrix used has endogenous analyte or interference	Check matrix ingredients for cross-reacting components (for example, interleukin modified tissue culture medium).
	Insufficient washes	Increase number of washes.

Problem	Probable Cause	Solution
Beads not in region or gate	Luminex® instrument not calibrated correctly or recently	Calibrate Luminex® instrument based on manufacturer's instructions, at least once a week or if temperature has changed by > 3 °C.
	Gate settings not adjusted correctly	Some Luminex® instruments (for example, Bio-Plex®) require different gate settings than those described in the kit protocol. Use instrument default settings.
	Wrong bead regions in protocol template	Check kit protocol for correct bead regions or analyte selection.
	Incorrect sample type used	Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required.
	Instrument not washed or primed	Prime the Luminex® instrument 4 times to rid it of air bubbles, wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid.
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 (for example, Bio-Plex®) default target setting for RP1 calibrator is set at high PMT. Use low target value for calibration and reanalyze plate.
	Plate incubation was too long with standard curve and samples	Use shorter incubation time.

Problem	Probable Cause	Solution
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

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 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.
Plate leaked	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
	Sample too viscous	May need to dilute sample.

Product Ordering

Replacement Reagents	Cat. No.
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 No	Cat. No.
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-1b 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-1a 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-TNFa Bead	77	MCYTNFA-MAG

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									

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