Mouse Bone Magnetic Bead Panel

96-Well Plate Assay

Cat. # MBNMAG-41K

MILLIPLEX® MAP

MOUSE BONE MAGNETIC BEAD PANEL KIT
96-Well Plate Assay

MBNMAG-41K

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By purchasing this product, which contains fluorescently labeled microsphere beads authorized by Luminex® Corporation ("Luminex®"), you, the customer, acquire the right under Luminex® Corporation's patent rights, if any, to use this product or any portion of this product, including without limitation the microsphere beads contained herein, only with Luminex® Corporation's laser based fluorescent analytical test instrumentation marketed under the name of Luminex® 100™ IS, Luminex® 200™, Luminex® HTS, FLEXMAP 3D®, MAGPIX®.

Mouse Bone Magnetic Bead Panel

INTRODUCTION

Bone metabolism is the dynamic process of ongoing bone deposition and resorption, controlled by osteoblasts, osteocytes, and osteoclasts. While osteoblasts and osteocytes (osteoblasts surrounded by matrix) are responsible for bone deposition, osteoclasts are responsible for bone resorption. Both are required to maintain bone structure, as well as an adequate supply of calcium. To maintain this metabolic balance these cells rely on complex signaling pathways involving hormones and cytokines to achieve the appropriate rates of growth and differentiation. The disruption of bone metabolism results in such disease as osteoporosis, osteoarthritis, rheumatoid arthritis, chronic kidney disease and bone metastases.

EMD Millipore recognizes the need to understand better the role that bone metabolism biomarkers play both in preserving normal bone structure and in the development of disease. 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 Human Bone Magnetic Bead panel enables you to focus on the therapeutic potential of bone metabolism**.** 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 BONE Magnetic Bead panel is the most versatile system available for bone metabolism research.

- MILLIPLEX MAP offers you the ability to:
  - Select a 10-plex or
  - 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 Bone Magnetic Bead kit is to be used for the simultaneous quantification from the following ACTH, IL-6, OPG, OC, Insulin, Leptin, DKK1, SOST, TNFα and FGF23.

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, 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 Bone Standard	MBN-8041	Lyophilized	1 vial
Mouse Bone Quality Controls 1 and 2	MBN-6041	Lyophilized	2 vials
Serum Matrix Note: Contains 0.08% Sodium Azide	LMC-SD	Lyophilized	1 vial
Set of one 96-Well Plate with 2 sealers	-----------	-----------	1 plate 2 sealers
Assay Buffer	L-AB1	30 mL	2 bottles
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	2 bottles
Mouse Bone Detection Antibody A	MBN-1041A	3.2 mL	1 bottle
Mouse Bone Detection Antibody B	MBN-1041B	5.5 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE	5.5 mL	1 bottle
Mixing Bottle (not provided with premixed panel)	-----------	-----------	1 bottle

Included Mouse Bone Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel.

Bead/Analyte Name	Luminex Bead Region	Customizable 10 Analytes (20X concentration, 200µL)	
		Available	Cat. #
Anti-ACTH Beads	12	✓	HACTH-MAG
Anti-IL6 Beads	26	✓	MIL6-MAG
Anti-OPG Beads	34	✓	M0PG-MAG
Anti-OC Beads	36	✓	M0C-MAG
Anti-Insulin Beads	37	✓	RMINS-MAG
Anti-Leptin Beads	38	✓	RMLPTN-MAG
Anti-DKK1 Beads	42	✓	MDKK1-MAG
Anti-SOST Beads	55	✓	MS0ST-MAG
Anti-TNFα Beads	65	✓	RMTNFA-MAG
Anti- FGF23 Beads	72	✓	MFGF23-MAG

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

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

Instrumentation / Materials

1. Adjustable Pipettes with Tips capable of delivering 25 µL to 1000 µL
2. Multichannel Pipettes capable of delivering 5 µL to 50 µL or 25 µL to 200 µL
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. Rubber Bands
6. Aluminum Foil
7. Absorbent Pads
8. Laboratory Vortex Mixer
9. Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
10. Titer Plate Shaker (Lab-Line Instruments Model #4625 or equivalent)
11. Luminex® 200™, HTS, FLEXMAP 3D®, or MAGPIX® with xPONENT® software by Luminex® Corporation
12. Automatic Plate washer for magnetic beads (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 hand held magnetic separation block for magnetic beads is not available, one can use a microtiter filter plate (EMD Millipore Catalog #MX-PLATE) to run the assay using a Vacuum Filtration Unit (EMD Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with EMD Millipore Vacuum Pump Catalog #WP6111560 or equivalent).

SAFETY PRECAUTIONS

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

TECHNICAL GUIDELINES

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

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at ≤ -20°C for 1 month and at ≤ -80°C for greater than one month.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8°C for up to one month.
- During the preparation of the standard curve, make certain to mix the higher concentration well before making the next dilution. Use a new tip with each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some analytes.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.
- When reading the assay on Luminex 200™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 4 alignment discs. When reading the assay on FLEXMAP 3D™, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 1 alignment disc. When reading the assay on MAGPIX, adjust probe height according to the protocols recommended by Luminex to the kit solid plate using 2 alignment discs.
- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells. If samples are diluted in assay buffer, use the assay buffer as matrix.
- For serum/plasma samples that require further dilution beyond 1:2, 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 in the Assay Buffer provided in the kit. For example, in a tube, 35 uL of serum may be combined with 35 uL of Assay Buffer. When further dilution beyond 1:2 is required, use Assay Buffer as the diluent.

For mouse Osteocalcin measurement, dilution of mouse serum samples in Assay Buffer at 1:20 -1:50 dilution is needed prior to the assay. Customers may need to determine the optimal dilution factors for their samples depending on the expected biological range. In general, serum samples from normal animals should be diluted ≥ 1:20 for quantification of mouse Osteocalcin. Assay Buffer provided in the kit should be used as the sample diluent. If the Osteocalcin concentrations for the diluted samples are between the highest and penultimate standard points, it is recommended that the samples be diluted further (e.g. at least 4-fold or greater dilution from the dilution factor used).

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.
- Serum samples should be diluted 1:2 in the Assay Buffer provided in the kit. For example, in a tube, 35 uL of serum may be combined with 35 uL of Assay Buffer. When further dilution beyond 1:2 is required, use Assay Buffer as the diluent.

SAMPLE COLLECTION AND STORAGE (continued)

- For mouse Osteocalcin measurement, dilution of mouse serum samples in Assay Buffer at 1:20 -1:50 dilution is needed prior to the assay. Customers may need to determine the optimal dilution factors for their samples depending on the expected biological range. In general, serum samples from normal animals should be diluted ≥ 1:20 for quantification of mouse Osteocalcin. Assay Buffer provided in the kit should be used as the sample diluent. If the Osteocalcin concentrations for the diluted samples are between the highest and penultimate standard points, it is recommended that the samples be diluted further (e.g. at least 4-fold or greater dilution from the dilution factor used).

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 1:2 freshly 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 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 3 antibody-immobilized beads, add 150 µL from each of the 3 bead sets to the Mixing Bottle. Then add 2.55 mL Assay Buffer.

Example 2: When using 4 antibody-immobilized beads, add 150 µL from each of the 4 bead sets to the Mixing Bottle. Then add 2.40 mL Assay Buffer.

B. Preparation of Quality Controls

Before use, reconstitute Quality Control 1 and Quality Control 2 with 250 µL deionized water. Invert the vial several times to mix and vortex. Allow the vial to sit for 5-10 minutes. Unused portion may be stored at ≤ -20°C for up to one month.

C. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 60 mL of 10X Wash Buffer (two bottles) with 540 mL deionized water. Store unused portion at 2-8°C for up to one month.

D. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

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

E. Preparation of Mouse Bone Standard

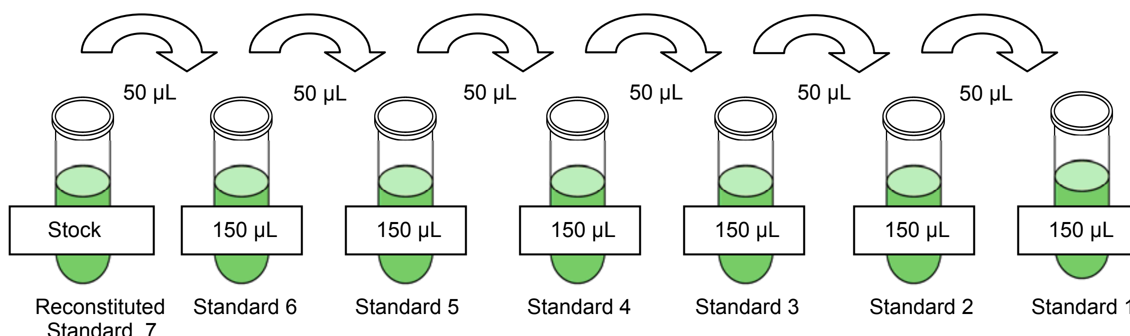
1.) Prior to use, reconstitute the Mouse Bone Standard with 250 µL deionized water to give a concentration described in the table below. Invert the vial several times to mix. Vortex the vial for 10 seconds. Allow the vial to sit for 5-10 minutes. This will be used as the Standard 7; the unused portion may be stored at ≤ -20°C for up to one month.

2). Preparation of Working Standards

Label six polypropylene microfuge tubes as Standard 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 1:4 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. The 0 Standard (Background) will be the Assay Buffer.

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

Standard Tube Number	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



Standard	ACTH (pg/mL)	IL-6 & FGF23 (pg/mL)	OPG (pg/mL)	OC & DKK1 (pg/mL)
Standard 1	4	7	16	15
Standard 2	15	29	63	59
Standard 3	59	117	254	234
Standard 4	234	469	1,016	938
Standard 5	938	1,875	4,063	3,750
Standard 6	3,750	7,500	16,250	15,000
Standard 7	15,000	30,000	65,000	60,000

Standard	Insulin (pg/mL)	Leptin (pg/mL)	SOST & TNF-α (pg/mL)
Standard 1	37	10	3
Standard 2	146	39	12
Standard 3	586	156	47
Standard 4	2,344	625	188
Standard 5	9,375	2,500	750
Standard 6	37,500	10,000	3,000
Standard 7	150,000	40,000	12,000

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, 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 the 0 pg/mL standard (Background).
4. Add 25 µL of Assay Buffer to the sample wells.
5. Add 25 µL of Sample (tissue culture supernatant or 1:2 or ≥1:20 diluted serum/plasma) into the appropriate wells.
6. Add 25 µL appropriate matrix solution to the background, standards, and control wells. When assaying tissue culture supernatant, use proper control tissue culture medium as the matrix solution. When assaying diluted serum or plasma, Serum Matrix provided in the kit as the matrix solution.
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.)

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

8. Add 25 µL of Mouse Bone Detection Antibody A or 25 µL of Assay Buffer to each well.
   a. When Osteocalcin or Insulin Beads are included in the analysis, add 25 µL of Mouse Bone Detection Antibody A to each well.
   b. If Osteocalcin or Insulin Beads are not in the analysis, add 25 µL of Assay Buffer 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 hours) at 4°C or incubate with agitation on a plate shaker 2 hours at RT (20-25°C).

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

11. Add 50 µL of Mouse Bone Panel Detection Antibody B into each well. (Note: Allow the Detection Antibody to warm to room temperature prior to addition.)

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

13. Add 50 µL Streptavidin-Phycoerythrin to each well containing the 50 µL of Detection Antibodies. (Note: Allow the SAPE to warm to room temperature prior to addition.)

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

Add 25 µL Detection Antibody A or Assay Buffer to each well.

Incubate overnight (16-18 hours) at 4°C or incubate 2 hours

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

Add 50 µL Mouse Bone Panel Detection Antibody B per well

Incubate 1 hour at RT

Do Not Aspirate

Add 50 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

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.18.

18. 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 100 µL Sheath 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 hand-held magnet or magnetic plate washer.

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

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

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

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

EQUIPMENT SETTINGS

Bio-Tek® ELx405:

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

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

1.) Soak program:

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

2.) Wash program:

Method:

1. Number of cycles: 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:

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.

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 10-Plex Beads	
	ACTH	12
	IL6	26
	OPG	34
	OC	36
	Insulin	37
	Leptin	38
	DKK1	42
	SOST	55
	TNFα	65
	FGF23	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 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 the Milliplex Analyst. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions.

Analyte	Overnight Protocol (n = 9 Assays)		2 Hour Protocol (n = 5 Assays)	
	MinDC (pg/mL)	MinDC+2SD (pg/mL)	MinDC (pg/mL)	MinDC+2SD (pg/mL)
ACTH	1.8	3.7	1.3	3.0
IL6	2.1	3.8	1.4	3.1
OPG	3.5	9.5	3.8	7.9
OC	6.6	9.0	4.7	8.9
Insulin	24.2	42.2	19.1	39.4
Leptin	3.3	7.3	2.6	5.6
DKK1	6.1	12.5	3.9	8.1
SOST	1.1	2.2	1.0	2.0
TNFα	1.0	2.4	0.7	1.7
FGF23	3.0	5.8	2.0	5.8

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		2 Hour Protocol
	Intra-assay %CV	Inter-assay %CV	Intra-assay %CV
ACTH	9	11	6
IL6	3	10	3
OPG	4	9	3
OC	3	11	3
Insulin	4	10	4
Leptin	4	11	3
DKK1	4	12	3
SOST	5	12	3
TNFα	5	9	3
FGF23	5	6	3

Accuracy

Spike Recovery: The data represent mean percent recovery of spiked standards ranging from low, medium, and high concentration in serum matrices (n=6).

Analyte	Overnight Protocol	2 Hour Protocol
	% Recovery in Serum Matrix	% Recovery in Serum Matrix
ACTH	81	90
IL6	92	90
OPG	97	94
OC	91	89
Insulin	91	93
Leptin	93	90
DKK1	88	88
SOST	91	88
TNFα	90	88
FGF23	91	84

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Insufficient Bead Count	Plate Washer aspirate height set too low	Adjust aspiration height according to manufacturers' instructions.
	Bead mix prepared inappropriately	Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting this into the plate.
	Samples cause interference due to particulate matter or viscosity	See above. Also sample probe may need to be cleaned with Alcohol flush, Back flush and washes; or if needed probe should be removed and sonicated.
	Probe height not adjusted correctly	When reading the assay on Luminex® 200™, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 4 alignment discs. When reading the assay on FLEXMAP 3D®, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 1 alignment disc. When reading the assay on MAGPIX®, adjust probe height according to the protocols recommended by Luminex® to the kit solid plate using 2 alignment discs.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately, and pipeting with Multichannel pipets without touching reagent in plate.
	Matrix used has endogenous analyte or interference	Check matrix ingredients for cross reacting components (e.g. interleukin modified tissue culture medium).
	Insufficient washes	Increase number of washes.
Beads not in region or gate	Luminex® not calibrated correctly or recently	Calibrate Luminex® based on Instrument Manufacturer's instructions, at least once a week or if temperature has changed by >3°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 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		
Filter plate will not vacuum	Vacuum pressure is insufficient Samples have insoluble particles High lipid concentration	Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. Centrifuge samples just prior to assay setup and use supernatant. 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 Bone Standard	MBN-8041
Mouse Bone Quality Controls 1 and 2	MBN-6041
Serum Matrix	LMC-SD
Mouse Bone Detection Antibody A	MBN-1041A
Mouse Bone Detection Antibody B	MBN-1041B
Streptavidin-Phycoerythrin	L-SAPE
Set of two 96-Well plates with sealers	MAG-PLATE
10X Wash Buffer	L-WB
Assay Buffer	L-AB1

Antibody-Immobilized Magnetic Beads

Analyte	*Bead #*	*Cat. #*
ACTH	12	HACTH-MAG
IL6	26	MIL6-MAG
OPG	34	M0PG-MAG
OC	36	M0C-MAG
Insulin	37	RMINS-MAG
Leptin	38	RMLPTN-MAG
DKK1	42	MDKK1-MAG
SOST	55	MS0ST-MAG
TNFα	65	RMTNFA-MAG
FGF23	72	MFGF23-MAG

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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
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WELL MAP

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