Mouse Bone Panel 2B

96 Well Plate Assay

Cat. # MBN2B-41K

MILLIPLEX® MAP

MOUSE BONE PANEL 2B KIT
96 Well Plate Assay

MBN2B-41K

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

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.

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. Therefore, we are proud to announce that the former LINCO*plex* Mouse Bone Metabolism Panel 2B now has the MILLIPLEX® MAP optimized format. While you will immediately recognize the quality and reproducibility that you have always trusted, you will also enjoy the enhancements that we have built into MILLIPLEX MAP.

Millipore's MILLIPLEX® MAP Mouse Bone Metabolism Panel 2B is to be used for the quantification of adenocorticotropic hormone (ACTH), insulin, IL-6, leptin, osteocalcin (OC), RANKL and TNFα in mouse serum, plasma, and cell/tissue culture supernatant samples. The panel provides biomedical researchers with quality tools for the study of bone metabolism related diseases.

This kit is for research purposes only.

Please read entire protocol before use.

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

PRINCIPLE

MILLIPLEX® MAP is based on the Luminex® xMAP® technology — one of the fastest growing and most respected multiplex technologies offering applications throughout the life sciences and capable of performing a variety of bioassays including immunoassays on the surface of fluorescent-coded beads known as 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.
- Once the standards and controls have been reconstituted, immediately transfer contents into polypropylene vials. **DO NOT STORE RECONSTITUTED STANDARDS OR CONTROLS IN GLASS VIALS**. For long-term storage, freeze reconstituted standards and controls at ≤ -20°C. Avoid multiple (>2) freeze/thaw cycles.
- **DO NOT FREEZE Antibody-Immobilized Beads, Detection Antibodies, and Streptavidin-Phycoerythrin**.

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

REAGENTS SUPPLIED	CATALOG NUMBER	VOLUME	QUANTITY
Mouse/Rat Bone Panel 2 Standard	LMBN-8041-2	lyophilized	1 vial
Mouse/Rat Bone Panel 2 Quality Controls	LMBN-6041-2	lyophilized	2 vials
Set of one 96-Well Filter Plate with 2 Sealers	MX-PLATE	----------	1 plate 2 sealers
Assay Buffer Note: Contains 0.08% Sodium Azide	L-AB1	30 mL	2 bottles
10X Wash Buffer Note: Contains 0.05% Proclin	L-WB	30 mL	1 bottle
Mouse Bone Detection Antibody A (Supplied only with orders containing Osteocalcin and / or Insulin beads)	LMBN-1041-A	3.2 mL	1 bottle
Mouse Bone Panel 2 Detection Antibody B (Supplied only with orders containing RANKL, Leptin, ACTH, IL-6 and /or TNFα beads)	LMBN-1041-B2	5.5 mL	1 bottle
Streptavidin-Phycoerythrin	L-SAPE	5.5 mL	1 bottle
Mixing Bottle	----------	----------	1 bottle

Included Mouse Bone Panel 2B Antibody-Immobilized Beads are dependent on customizable selection of analytes within the panel.

Mouse Bone Panel 2B Antibody-Immobilized Beads:

Bead/Analyte Name	Luminex Bead Region	Customizable 7 Analytes (20X concentration, 200µL) Available	Cat. #
Anti - Insulin Bead	05	✓	RME-INS
Anti – RANKL Bead	10	✓	MRANKL
Anti - Leptin Bead	16	✓	RME-LPTN
Anti – Osteocalcin	24	✓	MOC
Anti - ACTH Bead	48	✓	HPT-ACTH
Anti-IL-6 Bead	58	✓	MIL-6
Anti-TNFα Bead	64	✓	MTNF-A

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex Sheath Fluid (Luminex Catalogue #40-50000)

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. Absorbent Pads
7. Laboratory Vortex Mixer
8. Sonicator (Branson Ultrasonic Cleaner Model #B200 or equivalent)
9. Titer Plate Shaker (Lab-Line Instruments Model #4625 or equivalent)
10. Vacuum Filtration Unit (Millipore Vacuum Manifold Catalog #MSVMHTS00 or equivalent with Millipore Vacuum Pump Catalog #WP6111560 or equivalent)
11. Luminex 100™ IS, 200™, or HTS by Luminex Corporation
12. Plate Stand (Millipore Catalog # MX-STAND)

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. On disposal, flush with a large volume of water to prevent azide build up.

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.
- The bottom of the Microtiter Filter Plate should not be in direct contact with any surface during assay set-up or incubation times. The plate can be set on a

plate stand or on the non-flat side of the plate cover or any other plate holder to raise the plate from the surface. A plate stand can be purchased separately from Millipore (Millipore Catalog #MX-STAND).

- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- After the wash steps, keep the bottom of the Microtiter Filter Plate clean by blotting on paper towels or absorbent pads to prevent any leakage due to capillary action.
- Keep the vacuum suction on the plate as low as possible. It is recommended to have a vacuum setting that will remove 200 µL of buffer in $\geq$ 5 seconds (equivalent to < 100 mmHg).
- After hydration, all Standards and Controls must be transferred to polypropylene tubes.
- The Standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at ≤ -20°C for 1 month and at ≤ -80°C for greater than one month.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- Any unused mixed Antibody-Immobilized Beads may be stored in the 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. Adjust probe height according to the protocols recommended by Luminex to the kit filter plate using 3 alignment discs prior to reading an assay.
- 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, use the Serum Matrix provided in the kit.
- For cell/tissue homogenate, the final cell or tissue homogenate should be prepared in a buffer that has a neutral pH, contains minimal detergents or strong denaturing detergents, and has an ionic strength close to physiological concentration. Avoid debris, lipids, and cell/tissue aggregates. Centrifuge samples before use.

- Vortex all reagents well before adding to plate.

SAMPLE COLLECTION AND STORAGE

A. Preparation of Serum Samples:

- Allow the blood to clot for at least 30 minutes before centrifugation for 10 minutes at 1000xg. Remove serum and assay immediately or aliquot and store samples at ≤ -20°C.
- Avoid multiple (>2) freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.
- 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).
- Discard any unused serum samples.

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.
- For mouse Osteocalcin measurement, dilution of mouse plasma 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, plasma 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).
- Discard any unused plasma samples.

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.

NOTE:

- A maximum of 25 µL per well of cell / tissue culture supernatant samples or ≥ 1:20 freshly diluted serum or plasma samples can be used.
- All samples must be stored in polypropylene tubes. **DO NOT STORE SAMPLES IN GLASS.**
- Avoid debris, lipids and cells when using samples with gross hemolysis or lipemia.
- Care must be taken when using heparin as an anticoagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.
- Osteocalcin is sensitive to freeze/thaw cycles.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

Sonicate each antibody-bead vial for 30 seconds; vortex for 1 minute. Add 150 µL from each antibody bead vial to the Mixing Bottle and bring final volume to 3.0 mL with Assay Buffer. Vortex the mixed beads well. Unused portion may be stored at 2-8°C for up to one month.

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 5 antibody-immobilized beads, add 150 µL from each of the 5 bead sets to the Mixing Bottle. Then add 2.25 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 and then transfer the controls to appropriately labeled polypropylene microfuge tubes. Unused portion may be stored at ≤ -20°C for up to one month.

C. Preparation of Wash Buffer

Bring the 10X Wash Buffer to room temperature and mix to bring all salts into solution. Dilute 30 mL of 10X Wash Buffer with 270 mL deionized water. Store unused portion at 2-8°C for up to one month.

D. Preparation of Mouse/Rat Bone Panel 2 Standard

1.) Prior to use, reconstitute the lyophilized Mouse/Rat Bone Panel 2 Standard with 250 µL Deionized Water to give a concentration of 100,000 pg/mL for Insulin, a concentration of 40,000 pg/mL for RANKL, Osteocalcin and Leptin, and a concentration of 10,000 pg/mL for TNFα, IL-6 and ACTH. Invert the vial several times to mix. Allow the vial to set for 5-10 minutes to make sure that the standards are completely reconstituted, and then transfer the standard to an appropriately-labeled polypropylene microfuge tube. This stock solution will be used as the Standard 1. The unused portions of this stock may be stored at ≤ -20°C for up to one month.

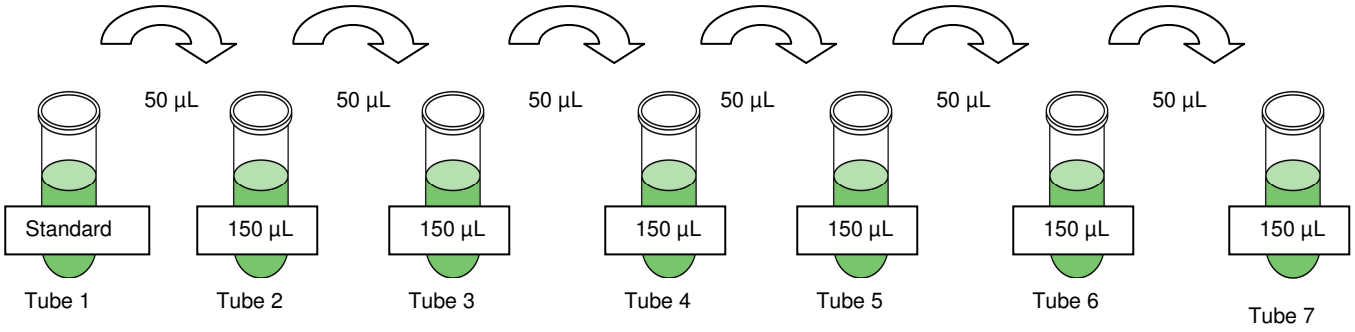
2.) Preparation of Working Standards

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

Tube Number	Standard Dilution	Volume of Deionized Water to Add	Volume of Standard to Add
1	Original	250 µL	0

Tube Number	Standard Dilution	Volume of Assay Buffer to Add	Volume of Standard to Add
2	1:4	150 µL	50 µL of Standard Tube 1 (Original Standard)
3	1:16	150 µL	50 µL of Standard Tube 2 (1:4 Standard)
4	1:64	150 µL	50 µL of Standard Tube 3 (1:16 Standard)
5	1:256	150 µL	50 µL of Standard Tube 4 (1:64 Standard)
6	1:1024	150 µL	50 µL of Standard Tube 5 (1:256 Standard)
7	1:4096	150 µL	50 µL of Standard Tube 6 (1:1024 Standard)

Preparation of Mouse/Rat Bone Panel 2 Standard



The serial dilutions result in the following concentrations of standards.

The Mouse/Rat Bone Panel 2 Standard Concentrations (pg/mL)

Tube Number	Standard Dilution	Insulin (pg/mL)	Osteocalcin RANKL Leptin (pg/mL)	TNFα IL-6 ACTH (pg/mL)
	Background	0	0	0
7	1:4096	24.4	9.8	2.4
6	1:1024	97.7	39.1	9.8
5	1:256	390.6	156.3	39.1
4	1:64	1,563	625	156.3
3	1:16	6,250	2,500	625
2	1:4	25,000	10,000	2,500
1	Original	100,000	40,000	10,000

IMMUNOASSAY PROCEDURE

- Prior to beginning this assay, it is imperative to read this protocol completely and to thoroughly understand the Technical Guidelines.
- Allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Diagram the placement of Standards [0 (Background), Tube 7, 6, 5, 4, 3, 2, 1], 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.
- 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. Prewet the filter plate by pipetting 200 µL of Assay Buffer into each well of the Microtiter Filter Plate. Seal and mix on a plate shaker for 10 minutes at room temperature (20-25°C).

2. Remove Assay Buffer by vacuum. **(NOTE: DO NOT INVERT PLATE.)** Blot excess Assay Buffer from the bottom of the plate with an absorbent pad or paper towels.

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 ≥1:20 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, use the Assay Buffer provided in the kit.

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

Vacuum

- 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 solution 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, cover it with the lid. Wrap a rubber band around the plate holder, plate and lid and incubate with agitation on a plate shaker overnight (16-20 hours) at 4°C
   (Note: A total volume of 100 µL should be in each well for the overnight incubation.)

10. Gently remove fluid by vacuum. **(NOTE: DO NOT INVERT PLATE.)**

11. Wash plate 3 times with 200 µL/well of Wash Buffer, removing Wash Buffer by vacuum filtration between each wash. Blot excess Wash Buffer from the bottom the plate by with an absorbent pad or paper towels.

12. Add 50 µL of Mouse Bone Panel 2 Detection Antibody B into each well. (Note: Allow the Detection Antibody to warm to room temperature prior to addition.)

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

14. Add 50 µL Streptavidin-Phycoerythrin to each well containing the 50 µL of Mouse Bone Panel 2 Detection Antibody B. (Note: Allow the SAPE to warm to room temperature prior to addition.)

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

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

Incubate overnight at 4°C with shaking

Vacuum and wash 3X with 200 µL Wash Buffer

Add 50 µL Mouse Bone Panel 2 Detection Antibody B per well

Incubate 1 hour at RT

Do Not Vacuum

Add 50 µL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT

16. Gently remove all contents by vacuum. **(NOTE: DO NOT INVERT PLATE.)**

17. Wash plate 3 times with 200 µL/well Wash Buffer, removing Wash Buffer by vacuum filtration between each wash. Wipe any excess buffer on the bottom of the plate with a tissue.

18. Add 100 µL of Sheath Fluid to all wells. Resuspend the beads on a plate shaker for 5 minutes.

19. Run plate on Luminex 100™ IS, 200™, or HTS.

20. Save the data and analyze the Median Fluorescent Intensity (MFI) using appropriate curve-fitting software. A weighted 5-parameter logistic method with weighting or cubic spline method is recommended.

Vacuum 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)

EQUIPMENT SETTINGS

These specifications are for the Luminex 100™ IS v.1.7 or Luminex 100™ IS v2.1/2.2, Luminex 200™ v2.3, xPONENT, and Luminex HTS. Luminex instruments with other software (e.g. MasterPlex, StarStation, LiquiChip, Bio-Plex, LABScan100) would need to follow instrument instructions for gate settings and additional specifications from the vendors.

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:	7-Plex Beads	
	Insulin	05
	RANKL	10
	Leptin	16
	Osteocalcin	24
	ACTH	48
	IL-6	58
	TNFα	64

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

ASSAY CHARACTERISTICS

Assay Sensitivities (minimum detectable concentrations, pg/mL)

MinDC: Minimum Detectable Concentration is calculated by the StatLIA® Immunoassay Analysis Software from Brendan Technologies. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions.

Analyte	MinDC (pg/mL)
Insulin	14.4
RANKL	2.7
Leptin	4.4
Osteocalcin	4.7
ACTH	1.3
IL-6	0.6
TNFα	0.6

Precision

Intra-assay precision is generated from the mean of the %CV from 16 reportable results across two different concentrations of analyte in a single assay. Inter-assay precision is generated from the mean of the %CV from 2 reportable results across 2 different concentrations of analyte across 6 different assays.

Analyte	Intra-Assay (CV%)	Inter-Assay (CV%)
Insulin	2.7	4.8
RANKL	3.1	11.0
Leptin	3.2	6.5
Osteocalcin	3.2	4.3
ACTH	2.9	7.5
IL-6	3.7	3.4
TNFα	2.5	9.3

Accuracy

Accuracy, defined as the percentage of measured analyte in samples spiked with known concentrations of analyte, was determined by taking the average recovery of 3 levels of analyte in 1:20 Assay Buffer diluted serum samples.

Analyte	% Recovery
Insulin	91
RANKL	78
Leptin	93
Osteocalcin	97
ACTH	85
IL-6	90
TNFα	93

Cross-Reactivity

There was no or negligible cross-reactivity between the antibodies and any of the other analytes in this panel.
Note: Mouse RANKL, ACTH, Leptin, and Insulin assays showed reactivity with rat samples.

TROUBLESHOOTING GUIDE

Problem	Probable Cause	Solution
Filter plate will not vacuum	Vacuum pressure is insufficient	Increase vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds.
	Samples have insoluble particles	Centrifuge samples just prior to assay set-up and use supernatant. If high lipid concentration, after centrifugation, remove lipid layer and use supernatant.
	Sample too viscous	May need to dilute sample.
Insufficient bead count	Vacuum pressure too high	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds.
	Bead mix prepared incorrectly	Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting 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, backflush and washes; or, if needed, probe should be removed and sonicated.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
Plate leaked	Vacuum pressure too high	Adjust vacuum pressure such that 0.2mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (prewetted) plate and continue.
	Plate set directly on table or absorbent towels during incubations or reagent additions	Set plate on plate stand 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 well.
	Probe height not adjusted correctly	Adjust probe to 3 alignment discs in well H6.
Background is too high	Background wells were contaminated	Avoid cross-well contamination by using sealer appropriately and by pipetting with multichannel pipets without touching reagent in plate.
	Matrix used has endogenous analyte or interference	Check matrix ingredients for crossreacting 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. 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 4 times to eliminate 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 vacuumed out, sensitivity may be low.
Low signal for standard curve	Detection Antibody may have been vacuumed out prior to adding Streptavidin Phycoerythrin	May need to repeat assay if desired sensitivity not achieved.
	Incubations done at incorrect 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 tech support for appropriate protocol modifications.
	Samples contain analyte concentrations higher than highest standard point	Samples may require dilution and reanalysis for that particular analyte.
	Standard curve was saturated at higher end of curve	See above.
High variation in	Multichannel pipet may not	Calibrate pipets.

samples and/or standards	be calibrated	
	Plate washing was not uniform	Confirm all reagents are vacuumed out 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 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.

REPLACEMENT REAGENTS **Catalog #**

Mouse/Rat Bone Panel 2 Standard	LMBN-8041-2
Mouse/Rat Bone Panel 2 Quality Controls	LMBN-6041-2
Mouse Bone Detection Antibody A	LMBN-1041-A
Mouse Bone Panel 2 Detection Antibody B	LMBN-1041-B2
Streptavidin-Phycoerythrin	L-SAPE
Assay Buffer	L-AB1
Set of two 96-Well Filter Plates with Sealers	MX-PLATE
10X Wash Buffer	L-WB

Antibody-Immobilized Beads

Analyte	Bead #	Catalog #
Insulin Bead	05	RME-INS
RANKL Bead	10	MRANKL
Leptin Bead	16	RME-LPTN
Osteocalcin Bead	24	MOC
ACTH Bead	48	HPT-ACTH
IL-6 Bead	58	MIL-6
TNFα Bead	64	MTNF-A

ORDERING INFORMATION

To place an order:

To assure the clarity of your custom Mouse Bone Panel 2B kit order, please FAX the following information to our customer service department:

- 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® Mouse Bone Analytes

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WELL MAP

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