



Rat RANKL Single Plex Magnetic Bead Kit

96-Well Plate Assay

Cat. # RRNKLMAG-31K-01

MILLIPLEX® MAP

**Rat RANKL SINGLE PLEX MAGNETIC BEAD KIT
96-Well Plate Assay**

RRNKL MAG-31K-01

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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®'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®'s laser based fluorescent analytical test instrumentation marketed under the name of Luminex® 100™ IS, 200™, HTS, FLEXMAP 3D®, MAGPIX®.

Rat RANKL Single Plex Magnetic Bead Kit

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. One of these proteins, Receptor Activator for Nuclear Factor kappa B Ligand (RANKL) activates osteoclasts and has been implicated in degenerative bone diseases such as rheumatoid arthritis and osteomyelitis. The balance between OPG and RANKL regulates osteoclast differentiation, activation and survival. Millipore recognizes the need to better understand the role that bone metabolism biomarkers like RANKL play both in preserving normal bone structure and in the development of disease. This single plex kit provides biomedical researchers using rat models with quality tools for the study of bone metabolism related diseases.

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

Each MILLIPLEX[®] MAP panel and kit includes:

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

In addition each panel and kit meets stringent manufacturing criteria to ensure batch-to-batch reproducibility. The MILLIPLEX[®] MAP Rat RANKL Single Plex Magnetic Bead Kit thus 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[®] MAP Rat RANKL Single Plex Magnetic Bead Kit is part of the most versatile system available for bone metabolism research. From our single to multiplex biomarker solutions, we partner with you to design, develop, analytically validate and build the most comprehensive library available for protein detection and quantitation.

MILLIPLEX[®] MAP offers you 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.

MILLIPLEX[®] MAP Rat RANKL Single Plex Magnetic Bead Kit is to be used for the quantification of RANKL in rat serum, plasma or tissue culture samples.

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

Please read entire protocol before use.

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

PRINCIPLE

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

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

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

STORAGE CONDITIONS UPON RECEIPT

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

REAGENTS SUPPLIED

Note: Store all reagents at 2 – 8°C

| Reagents Supplied | Catalog Number | Volume | Quantity |
|---|-----------------|-------------|--|
| RANKL Standard | RBN-8031-RNKL | Lyophilized | 1 vial |
| RANKL Quality Controls 1 and 2 | RBN-6031-RNKL | Lyophilized | 2 vials |
| Serum Matrix Note: Contains 0.08% Sodium Azide | LMC-SD | Lyophilized | 1 bottle (required for serum and plasma samples only) |
| Set of one 96-Well Plate with 2 sealers | ----- | ----- - | 1 plate 2 sealers |
| Assay Buffer | L-AB1 | 30 mL | 1 bottle |
| 10X Wash Buffer Note: Contains 0.05% Proclin | L-WB | 60 mL | 1 bottle |
| RANKL Detection Antibodies | LMBN-1041-RANKL | 5.5 mL | 1 bottle |
| Streptavidin-Phycoerythrin | L-SAPE5 | 5.5 mL | 1 bottle |

RANKL Single Plex Antibody-Immobilized Magnetic Beads:

| Bead/Analyte Name | Luminex® Magnetic Bead Region | (1X concentration, 3.5 mL) Available Cat. # | |
|-------------------|--|---|-----------|
| Anti -RANKL Bead | 54 | ✓ | MRNKL-MAG |

MATERIALS REQUIRED BUT NOT PROVIDED

Reagents

1. Luminex[®] Sheath Fluid (EMD Millipore Catalog # SHEATHFLUID) or Luminex[®] Drive Fluid (EMD Millipore Catalog # MPXDF-4PK)

Instrumentation / Materials


1. Adjustable Pipettes with Tips capable of delivering 25 µL to 1000 µL
2. Multichannel Pipettes capable of delivering 5 µL to 50 µL or 25 µL to 200 µL
3. Reagent Reservoirs
4. Polypropylene Microfuge Tubes
5. Rubber Bands
6. Aluminum Foil
7. Absorbent Pads
8. Laboratory Vortex Mixer
9. Sonicator (Branson Ultrasonic Cleaner Model # B200 or equivalent)
10. Titer Plate Shaker (VWR[®] Microplate Shaker Cat # 12620-926 or equivalent)
11. Luminex[®] 200™, HTS, FLEXMAP 3D[®], or MAGPIX[®] with xPONENT[®] software by Luminex[®] Corporation
12. Automatic Plate Washer for magnetic beads (BioTek[®] 405 LS and 405 TS, EMD Millipore Catalog # 40-094, # 40-095, # 40-096, # 40-097 or equivalent) or Handheld Magnetic Separation Block (EMD Millipore Catalog # 40-285 or equivalent).

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






SAFETY PRECAUTIONS

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

Note: See Full Labels of Hazardous components.

| Ingredient, Cat # | | Full Label | |
|----------------------------|--------|---|---|
| Streptavidin-Phycoerythrin | L-SAPE |  | Warning. Causes serious eye irritation. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. |

Full Labels of Hazardous components (continued)

| Ingredient, Cat # | | Full Label | |
|---------------------|-----------------|---|--|
| 10X Wash Buffer | L-WB |  | <p>Warning. May cause an allergic skin reaction. Wear protective gloves. IF ON SKIN: Wash with plenty of soap and water.</p> |
| Rat RANKL QC1 & QC2 | RBN-6031-RNKL |  | <p>Warning. Harmful if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment.</p> |
| Rat RANKL Standard | RBN-8031-RNKL |  | <p>Warning. Harmful if swallowed. Toxic to aquatic life with long lasting effects. Avoid release to the environment.</p> |
| Serum Matrix | LMC-SD | No Symbol Required | <p>Harmful to aquatic life with long lasting effects. Avoid release to the environment.</p> |
| Assay Buffer | L-AB1 |  | <p>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.</p> |
| Detection Antibody | LMBN-1041-RANKL |  | <p>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.</p> |

TECHNICAL GUIDELINES

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

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not use beyond the expiration date on the label.
- Do not mix or substitute reagents with those from other lots or sources.
- The Antibody-Immobilized Beads are light sensitive and must be protected from light at all times. Cover the assay plate containing beads with opaque plate lid or aluminum foil during all incubation steps.
- It is important to allow all reagents to warm to room temperature (20-25°C) before use in the assay.
- Incomplete washing can adversely affect the assay outcome. All washing must be performed with the Wash Buffer provided.
- The standards prepared by serial dilution must be used within 1 hour of preparation. Discard any unused standards except the standard stock which may be stored at $\leq -20^{\circ}\text{C}$ for 1 month and at $\leq -80^{\circ}\text{C}$ for greater than one month.
- If samples fall outside the dynamic range of the assay, further dilute the samples with the appropriate diluent and repeat the assay.
- Any unused mixed Antibody-Immobilized Beads may be stored in the Mixing Bottle at 2-8°C for up to one month.
- During the preparation of the standard curve, make certain to mix the higher concentration well before making the next dilution. Use a new tip with each dilution.
- The plate should be read immediately after the assay is finished. If, however, the plate cannot be read immediately, seal the plate, cover with aluminum foil or an opaque lid, and store the plate at 2-8°C for up to 24 hours. Prior to reading, agitate the plate on the plate shaker at room temperature for 10 minutes. Delay in reading a plate may result in decreased sensitivity for some analytes.
- The titer plate shaker should be set at a speed to provide maximum orbital mixing without splashing of liquid outside the wells. For the recommended plate shaker, this would be a setting of 5-7 which is approximately 500-800 rpm.
- Ensure that the needle probe is clean. This may be achieved by sonication and/or alcohol flushes.
- When reading the assay on Luminex[®] 200[™], adjust probe height according to the protocols recommended by Luminex[®] to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX[®], adjust probe height according to the protocols recommended by Luminex[®] to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D[®], adjust probe height according to the protocols recommended by Luminex[®] to the kit solid plate using 1 alignment disc.
For FLEXMAP 3D[®] when using the solid plate in the kit, the final resuspension should be with 150 μL Sheath Fluid in each well and 75 μL should be aspirated.
- For cell culture supernatants or tissue extraction, use the culture or extraction medium as the matrix solution in background, standard curve and control wells. If samples are diluted in assay buffer, use the assay buffer as matrix.

TECHNICAL GUIDELINES (continued)

- For serum/plasma samples that require 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.
- 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 $\leq -20^{\circ}\text{C}$.
- Avoid multiple > 2 freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.

B. Preparation of Plasma Samples:

- Plasma collection using EDTA as an anti-coagulant is recommended. Centrifuge for 10 minutes at 1000xg within 30 minutes of blood collection. Remove plasma and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$.
- Avoid multiple > 2 freeze/thaw cycles.
- When using frozen samples, it is recommended to thaw the samples completely, mix well by vortexing and centrifuge prior to use in the assay to remove particulates.

C. Preparation of Tissue Culture Supernatant:

- Centrifuge the sample to remove debris and assay immediately or aliquot and store samples at $\leq -20^{\circ}\text{C}$.
- 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 neat serum or plasma can be used. Tissue culture or other media may also be used.
- All samples must be stored in polypropylene tubes. **DO NOT STORE SAMPLES IN GLASS.**
- Avoid debris, lipids and cells when using samples with gross hemolysis or lipemia.
- Care must be taken when using heparin as an anti-coagulant since an excess of heparin will provide falsely high values. Use no more than 10 IU heparin per mL of blood collected.

PREPARATION OF REAGENTS FOR IMMUNOASSAY

A. Preparation of Antibody-Immobilized Beads

Sonicate the antibody-bead vial for 30 seconds; vortex for 1 minute. 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.)

B. Preparation of Quality Controls

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

C. Preparation of Wash Buffer

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

D. Preparation of Serum Matrix

This step is required for serum or plasma samples only.

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

E. Preparation of RANKL Standard

1.) Prior to use, reconstitute the RANKL Standard with 250 µL deionized water to give a 15,000 pg/mL concentration of RANKL. 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 15,000 pg/mL standard; the unused portion may be stored at ≤ -20°C for up to one month.

2). Preparation of Working Standards

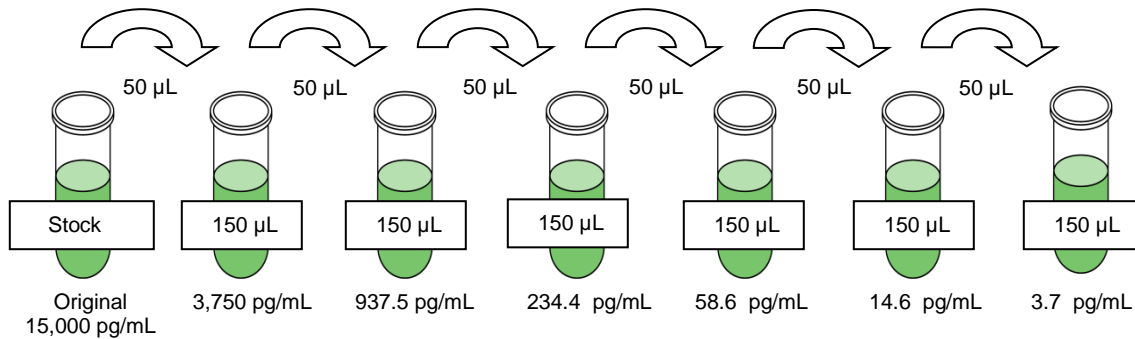
Label six polypropylene microfuge tubes as 3,750, 937.5, 234.4, 58.6, 14.6, and 3.7 pg/mL. Add 150 µL of Assay Buffer to each of the six tubes. Prepare serial dilutions by adding 50 µL of the reconstituted 15,000 pg/mL standard to the 3,750 pg/mL tube, mix well and transfer 50 µL of the 3,750 pg/mL standard to the 937.5 pg/mL tube, mix well and transfer 50 µL of the 937.5 pg/mL standard to the 234.4 pg/mL tube, mix well and transfer 50 µL of the 234.4 pg/mL standard to the 58.6 pg/mL tube, mix well and transfer 50 µL of the 58.6 pg/mL standard to the 14.6 pg/mL tube, mix well and transfer 50 µL of the 14.6 pg/mL standard to the 3.7 pg/mL tube and mix well. The 0 pg/mL standard (Background) will be the Assay Buffer.

PREPARATION OF REAGENTS FOR IMMUNOASSAY (continued)

| Standard Concentration pg/mL | Volume of Deionized Water to Add | Volume of Standard to Add |
|---------------------------------|--|------------------------------|
| Original 15,000 pg/mL | 250 µL | 0 |

| Standard Concentration (pg/mL) | Volume of Assay Buffer to Add | Volume of Standard to Add |
|-----------------------------------|----------------------------------|------------------------------|
| 3,750 | 150 µL | 50 µL of 15,000 pg/mL |
| 937.5 | 150 µL | 50 µL of 3,750 pg/mL |
| 234.4 | 150 µL | 50 µL of 937.5 pg/mL |
| 58.6 | 150 µL | 50 µL of 234.4 pg/ml |
| 14.6 | 150 µL | 50 µL of 58.6 pg/mL |
| 3.7 | 150 µL | 50 µL of 14.6 pg/mL |

Preparation of Standards Example 1 – 7 tubes



| Standard | RANKL (pg/mL) |
|------------|------------------|
| Standard 1 | 3.7 |
| Standard 2 | 14.6 |
| Standard 3 | 58.6 |
| Standard 4 | 234.4 |
| Standard 5 | 937.5 |
| Standard 6 | 3,750 |
| Standard 7 | 15,000 |

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), 3.7, 14.6, 58.6, 234.4, 937.5, 3,750, 15,000 pg/mL, Controls 1 and 2, and Samples on Well Map Worksheet in a vertical configuration. (Note: Most instruments will only read the 96-well plate vertically by default.) It is recommended to run the assay in duplicate.
- If using a filter plate, set the filter plate on a plate holder at all times during reagent dispensing and incubation steps so that the bottom of the plate does not touch any surface.

1. Add 200 µL of 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 provided in the kit. When assaying tissue culture or other supernatant, use proper control culture medium as the matrix solution.
6. Add 25 µL of Sample (neat) into the appropriate wells.
7. Vortex Mixing Bottle and add 25 µL of the Mixed or Premixed Beads to each well. (Note: During addition of Beads, shake bead bottle intermittently to avoid settling.)
8. Seal the plate with a plate sealer. Wrap the plate with foil and incubate with agitation on a plate shaker for 16-20 hours at 4°C.

Add 200 µL Assay Buffer per well



Shake 10 min, RT

Decant

- Add 25 µL Standard or Control to appropriate wells
- Add 25 µL Assay Buffer to background and sample wells
- Add 25 µL appropriate matrix solution to background, standards, and control wells
- Add 25 µL [neat] Samples to sample wells
- Add 25 µL Beads to each well



Incubate 16-20 hours at 4°C

9. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.
10. Add 50 μL of Detection Antibodies into each well. (Note: Allow the Detection Antibodies to warm to room temperature prior to addition.)
11. Seal, cover with foil and incubate with agitation on a plate shaker for 1 hour at room temperature (20-25°C). **DO NOT ASPIRATE AFTER INCUBATION.**
12. Add 50 μL Streptavidin-Phycoerythrin to each well containing the 50 μL of Detection Antibodies.
13. Seal, cover with foil and incubate with agitation on a plate shaker for 30 minutes at room temperature (20-25°C).
14. Gently remove well contents and wash plate 3 times following instructions listed in the **PLATE WASHING** section.
15. Add 100 μL of Sheath Fluid (or Drive Fluid if using MAGPIX®) to all wells. Resuspend the beads on a plate shaker for 5 minutes.
16. Run plate on Luminex® 200™, HTS, FLEXMAP 3D® or MAGPIX® with xPONENT® software.
17. Save and analyze the Median Fluorescent Intensity (MFI) data using a 5-parameter logistic or spline curve-fitting method for calculating analyte concentrations in samples. (Note: For diluted samples, multiply the calculated concentration by the dilution factor.)



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

Add 50 μL Detection Antibodies per well



Incubate 1 hour at RT

Do Not Aspirate

Add 50 μL Streptavidin-Phycoerythrin per well

Incubate for 30 minutes at RT



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

Add 100 μL Sheath Fluid or Drive Fluid per well

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

PLATE WASHING

1.) Solid Plate

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

- A.) Handheld magnet (EMD Millipore Catalog # 40-285) - Rest plate on magnet for 60 seconds to allow complete settling of magnetic beads. Remove well contents by gently decanting the plate in an appropriate waste receptacle and gently tapping on absorbent pads to remove residual liquid. Wash plate with 200 μ L of Wash Buffer by removing plate from magnet, adding Wash Buffer, shaking for 30 seconds, reattaching to magnet, letting beads settle for 60 seconds and removing well contents as previously described after each wash. Repeat wash steps as recommended in Assay Procedure.
- B.) Magnetic plate washer (EMD Millipore Catalog # 40-094, # 40-095, # 40-096 and # 40-097) - Please refer to specific automatic plate washer manual for appropriate equipment settings. Please note that after the final aspiration, there will be approximately 25 μ L of residual wash buffer in each well. This is expected when using the BioTek[®] plate washer and this volume does not need to be aspirated from the plate.

If using an automatic plate washer other than BioTek[®] 405 LS or 405 TS, please refer to the manufacturer's recommendations for programming instructions.

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

If using a filter plate, use a vacuum filtration manifold to remove well contents. Wash plate with 200 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[®], and MAGPIX[®] with xPONENT[®] software:

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

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

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

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

EQUIPMENT SETTINGS (continued)

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

| | | |
|----------------|-------------------|----|
| Events: | 50, per bead | |
| Sample Size: | 50 µL | |
| Gate Settings: | 8,000 to 15,000 | |
| Reporter Gain: | Default (low PMT) | |
| Time Out: | 60 seconds | |
| Bead Set: | | |
| | RANKL | 54 |

QUALITY CONTROLS

The ranges for each analyte in Quality Control 1 and 2 are provided on the card insert or can be located at the EMD Millipore website emdmillipore.com using the catalog number as the keyword.

ASSAY CHARACTERISTICS

Cross-Reactivity

Rat RANKL assay shows reactivity with recombinant mouse RANKL.

Assay Sensitivities (minimum detectable concentrations, pg/mL)

Minimum Detectable Concentration (MinDC) is calculated using MILLIPLEX[®] Analyst 5.1. It measures the true limits of detection for an assay by mathematically determining what the empirical MinDC would be if an infinite number of standard concentrations were run for the assay under the same conditions.

| Analyte | Overnight Protocol (n = 5 Assays) | | 2 Hour Protocol (n = 3 Assays) | |
|---------|--------------------------------------|----------------------|--------------------------------|----------------------|
| | MinDC (pg/mL) | MinDC+2SD (pg/mL) | MinDC (pg/mL) | MinDC+2SD (pg/mL) |
| RANKL | 1 | 2.6 | 1 | 1.4 |

Precision

Intra-assay precision is generated from the mean of the %CV's from 8 reportable results across two different concentrations of analytes in a single assay. Inter-assay precision is generated from the mean of the %CV's across two different concentrations of analytes across 4 different assays.

| Analyte | Overnight Protocol | |
|---------|--------------------|-----------------|
| | Intra-assay %CV | Inter-assay %CV |
| RANKL | < 10 | < 15 |

Accuracy

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

| Analyte | Overnight Protocol |
|---------|----------------------------|
| | % Recovery in Serum Matrix |
| RANKL | 99 |

TROUBLESHOOTING GUIDE

| Problem | Probable Cause | Solution |
|-----------------------------|--|---|
| Insufficient bead count | Plate washer aspirate height set too low | Adjust aspiration height according to manufacturers' instructions. |
| | Bead mix prepared inappropriately | Sonicate bead vials and vortex just prior to adding to bead mix bottle according to protocol. Agitate bead mix intermittently in reservoir while pipetting this into the plate. |
| | Samples cause interference due to particulate matter or viscosity | See above. Also sample probe may need to be cleaned with alcohol flushes, back flushes and washes; or, if needed, probe should be removed and sonicated. |
| | Probe height not adjusted correctly | When reading the assay on Luminex [®] 200™, adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 3 alignment discs. When reading the assay on MAGPIX [®] , adjust probe height to the kit solid plate or to the recommended EMD Millipore filter plates using 2 alignment discs. When reading the assay on FLEXMAP 3D [®] , adjust probe height to the kit solid plate using 1 alignment disc. For FLEXMAP 3D [®] when using the solid plate in the kit, the final resuspension should be with 150 µL Sheath Fluid in each well and 75 µL should be aspirated. |
| Background is too high | Background wells were contaminated | Avoid cross-well contamination by using sealer appropriately and pipetting with multichannel pipettes without touching reagent in plate. |
| | Matrix used has endogenous analyte or interference | Check matrix ingredients for cross-reacting components (e.g. interleukin modified tissue culture medium). |
| | Insufficient washes | Increase number of washes. |
| Beads not in region or gate | Luminex [®] instrument not calibrated correctly or recently | Calibrate Luminex [®] instrument based on manufacturer's instructions, at least once a week or if temperature has changed by >3°C. |
| | Gate settings not adjusted correctly | Some Luminex [®] instruments (e.g. Bio-Plex [®]) require different gate settings than those described in the kit protocol. Use instrument default settings. |
| | Wrong bead regions in protocol template | Check kit protocol for correct bead regions or analyte selection. |
| | Incorrect sample type used | Samples containing organic solvents or if highly viscous should be diluted or dialyzed as required. |
| | Instrument not washed or primed | Prime the Luminex [®] instrument 4 times to rid it of air bubbles, wash 4 times with sheath fluid or water if there is any remnant alcohol or sanitizing liquid. |

| Problem | Probable Cause | Solution |
|---|---|---|
| Beads not in region or gate (cont.) | 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® 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. 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 technical 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 pipette 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 pipettes. Confirm all reagents are removed completely in all wash steps. See above. Plate should be agitated during all incubation steps using an orbital plate shaker at a speed where beads are in constant motion without causing splashing. Check when reusing plate sealer that no reagent has touched sealer. Care should be taken when using same pipette tips that are used for reagent additions and that pipette tip does not touch reagent in plate. |

| FOR FILTER PLATES ONLY | | |
|------------------------------|---|--|
| Problem | Probable Cause | Solution |
| Filter plate will not vacuum | Vacuum pressure is insufficient | Increase vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. |
| | Samples have insoluble particles | Centrifuge samples just prior to assay set-up and use supernatant. |
| | High lipid concentration | After centrifugation, remove lipid layer and use supernatant. |
| Plate leaked | Vacuum pressure too high | Adjust vacuum pressure such that 0.2 mL buffer can be suctioned in 3-5 seconds. May need to transfer contents to a new (blocked) plate and continue. |
| | Plate set directly on table or absorbent towels during incubations or reagent additions | Set plate on plate holder or raised edge so bottom of filter is not touching any surface. |
| | Insufficient blotting of filter plate bottom causing wicking | Blot the bottom of the filter plate well with absorbent towels after each wash step. |
| | Pipette touching plate filter during additions | Pipette to the side of plate. |
| | Probe height not adjusted correctly | Adjust probe to 3 alignment discs in well H6. |
| | Sample too viscous | May need to dilute sample. |

REPLACEMENT REAGENTS

Catalog

| | |
|--|-----------------|
| RANKL Standard | RBN-8031-RNKL |
| RANKL Quality Controls 1 and 2 | RBN-6031-RNKL |
| Serum Matrix | LMC-SD |
| RANKL Detection Antibodies | LMBN-1041-RANKL |
| Streptavidin-Phycoerythrin | L-SAPE5 |
| Assay Buffer | L-AB1 |
| Set of two 96-Well plates with sealers | MAG-PLATE |
| 10X Wash Buffer | L-WB |

Antibody-Immobilized Magnetic Beads

| | | |
|----------------|---------------|---------------|
| <u>Analyte</u> | <u>Bead #</u> | <u>Cat. #</u> |
| RANKL Bead | 54 | MRNKL-MAG |

ORDERING INFORMATION

To place an order or to obtain additional information about our immunoassay products, please contact your Customer Service or Technical Support Specialist.

Contact information for each region can be found on our website:

emdmillipore.com/contact

Conditions of Sale

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

Safety Data Sheets (SDS)

Safety Data Sheets for EMD Millipore products may be downloaded through our website at emdmillipore.com/msds.

WELL MAP

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|--|-----------------------------|-----------------|------|---|---|---|---|---|----|----|----|
| A | 0 pg/mL Standard (Background) | 234.4 pg/mL Standard | QC-1 Control | Etc. | | | | | | | | |
| B | 0 pg/mL Standard (Background) | 234.4 pg/mL Standard | QC-1 Control | | | | | | | | | |
| C | 3.7 pg/mL Standard | 937.5 pg/mL Standard | QC-2 Control | | | | | | | | | |
| D | 3.7 pg/mL Standard | 937.5 pg/mL Standard | QC-2 Control | | | | | | | | | |
| E | 14.6 pg/mL Standard | 3,750 pg/mL Standard | Sample 1 | | | | | | | | | |
| F | 14.6 pg/mL Standard | 3,750 pg/mL Standard | Sample 1 | | | | | | | | | |
| G | 58.6 pg/mL Standard | 15,000 pg/mL Standard | Sample 2 | | | | | | | | | |
| H | 58.6 pg/mL Standard | 15,000 pg/mL Standard | Sample 2 | | | | | | | | | |