Magnetic Luminex® Performance Assay

Human MMP Premixed Kit

Catalog Number FCSTM07

For the simultaneous quantitative determination of multiple human matrix metalloproteinase (MMP) concentrations in cell culture supernates, serum, plasma, platelet-poor plasma, saliva, and urine.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

The matrix metalloproteinases (MMPs) consist of 24 known human zinc proteases with essential roles in breaking down components of the extracellular matrix (ECM) (1-5). In addition to ECM proteins, other potential MMP substrates include cytokines (6-10), chemokines (11), growth factors and binding proteins (12-15), cell/cell adhesion molecules (16), and other proteinases (17, 18). With a few exceptions, MMPs share common structural motifs including a pro-peptide domain, a catalytic domain, a hinge region, and a hemopexin-like domain (2, 4, 5). Synthesized as pro-enzymes, most are secreted before conversion to their active forms. In general, the activation mechanism is thought to occur in a stepwise fashion involving disruption of the interaction between the catalytic site zinc and a cysteine-thiol group in the pro-peptide domain. This is followed by cleavage of the pro-peptide (5). Activation can be mediated by several serine proteases (19-21), MMPs (4, 17, 21-22), or potentially via NO-mediated S-nitrosylation of the pro-peptide cysteine-thiol group (23). In some cases, activation can take place intracellularly via a furin-like serine protease (24, 25). MMPs are expressed by many cell types and can be upregulated in response to adhesion molecules, growth factors, cytokines, and hormones (2-5). They have been implicated in several physiological processes including tissue morphogenesis (26-28), cell migration (29-31), wound healing (32), bone remodeling (33, 34), and angiogenesis (35-37). MMP activities are modulated on several levels including transcription, pro-enzyme activation, or by their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs) (5, 38). Imbalances in MMP regulation have been implicated in several pathological processes including cancer (39, 40), cardiovascular disorders (41, 42), and arthritis (43-45).

This kit can be used to simultaneously assess the levels of multiple MMPs in a single sample. For ease of use, the microparticles and the biotinylated detection antibodies are premixed in respective vials.

Analyte	Microparticle Region	Performance Data Online (www.RnDSystems.com/pdf/...)
EMMPRIN	30	LMPM972.pdf
MMP-1	20	LMPM901.pdf
MMP-2	19	LMPM902.pdf
MMP-3	21	LMPM513.pdf
MMP-7	22	LMPM907.pdf
MMP-8	25	LMPM908.pdf
MMP-9	26	LMPM911.pdf
MMP-10	27	LMPM910.pdf
MMP-12	28	LMPM919.pdf
MMP-13	29	LMPM511.pdf

PRINCIPLE OF THE ASSAY

Magnetic Luminex® Performance Assay multiplex kits are designed for use with the Luminex® MAGPIX® CCD Imager. Alternatively, kits can be used with the Luminex® 100/200™, Luminex® FLEXMAP 3D®, or Bio-Rad® Bio-Plex®, dual laser, flow-based sorting and detection platforms.

Analyte-specific antibodies are pre-coated onto magnetic microparticles embedded with fluorophores at set ratios for each unique microparticle region. Microparticles, standards and samples are pipetted into wells and the immobilized antibodies bind the analytes of interest. After washing away any unbound substances, a biotinylated antibody cocktail specific to the analytes of interest is added to each well. Following a wash to remove any unbound biotinylated antibody, streptavidin-phycoerythrin conjugate (Streptavidin-PE), which binds to the biotinylated antibody, is added to each well. Final washes remove unbound Streptavidin-PE, the microparticles are resuspended in buffer and read using the Luminex® MAGPIX® Analyzer. A magnet in the analyzer captures and holds the superparamagnetic microparticles in a monolayer. Two spectrally distinct Light Emitting Diodes (LEDs) illuminate the microparticles. One LED excites the dyes inside each microparticle to identify the region and the second LED excites the PE to measure the amount of analyte bound to the microparticle. A sample from each well is imaged with a CCD camera with a set of filters to differentiate excitation levels.

Analysis with the Luminex® 100/200™, Luminex® FLEXMAP 3D®, or Bio-Rad Bio-Plex uses one laser to excite the dyes inside each microparticle to identify the microparticle region and the second laser to excite the PE to measure the amount of analyte bound to the microparticle. All fluorescence emissions from each microparticle as it passes through the flow cell is then analyzed to differentiate emission levels using a Photomultiplier Tube (PMT) and an Avalanche Photodiode.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples fall outside the dynamic range of the assay, further dilute the samples with calibrator diluent and repeat the assay.
- Any variation in diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until these factors have been tested in the Magnetic Luminex® Performance Assay, the possibility of interference cannot be excluded.
- Magnetic Luminex® Performance Assays afford the user the benefit of multianalyte analysis of biomarkers in a single complex sample. For each sample type, a single multipurpose diluent is used to optimize recovery, linearity, and reproducibility. Such a multipurpose diluent may not optimize any single analyte to the same degree that a unique diluent selected for analysis of that analyte can optimize conditions. Therefore, some performance characteristics may be more variable than those for assays designed specifically for single analyte analysis.
- **Only the analytes listed on the Standard Value Card can be measured with this kit. Refer to the enclosed certificate of analysis for specific analytes included in this kit.**

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED, DILUTED, OR RECONSTITUTED MATERIAL
MMP Standard Cocktail	894339	2 vials of recombinant human MMPs in a buffered protein base with preservatives; lyophilized.	Discard after use. Use a fresh standard for each assay.
Human MMP Premixed Kit Magnetic Microparticle Cocktail	894538	1.2 mL of a concentrated microparticle cocktail with preservatives.	May be stored for up to 1 month at 2-8 °C.* *Once diluted, 1X solutions must be discarded. Use fresh diluents for each assay.*
Human MMP Premixed Kit Biotin-Ab Cocktail	894112	1.2 mL of a concentrated biotinylated antibody cocktail with preservatives.	
Streptavidin-PE	892525	0.07 mL of a concentrated streptavidin-phycoerythrin conjugate with preservatives.	
Microparticle Diluent 3	895857	6 mL of a buffered protein base with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Biotin Antibody Diluent 2	895832	5.5 mL of a buffered protein base with preservative.	
Calibrator Diluent RD5-37	895853	21 mL of buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Microplate	641385	1 flat-bottomed 96-well microplate used as a vessel for the assay.	
Mixing Bottles	895505	2 empty 8 mL bottles used for mixing microparticles with Microparticle Diluent.	
Plate Sealers	640445	4 adhesive foil strips.	
Standard Value Card	749407	1 card listing the Standard Cocktail reconstitution volume and working standard concentrations for this kit lot.	

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Luminex® MAGPIX®, Luminex® 100/200™, Luminex® FLEXMAP 3D®, or Bio-Rad Bio-Plex analyzer with X-Y platform.
- Hand-held microplate magnet or platewasher with a magnetic platform.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Multi-channel pipette, manifold dispenser, or automated dispensing unit.
- 500 mL graduated cylinder.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 800 ± 50 rpm.
- Microcentrifuge.
- **Polypropylene** test tubes for dilution of standards and samples.
- Luminex® Performance Assay Controls (optional; R&D Systems®, Catalog # QC12).

PRECAUTIONS

MMPs are detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Protect microparticles and Streptavidin-PE from light at all times to prevent photobleaching.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Cell culture supernate samples are not suitable for use in the MMP-2 assay.*

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifuging for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Platelet-poor Plasma - Collect plasma on ice using EDTA or heparin as an anticoagulant. Centrifuge at 2-8 °C for 15 minutes at 1000 x g within 30 minutes of collection. For more complete platelet removal, an additional centrifugation step of the separated plasma at 1500 x g for 10 minutes at 2-8 °C is recommended. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Plasma and platelet-poor plasma samples are not suitable for use in the MMP-13 assay. EDTA and Citrate are not recommended for use in this assay due to their chelating properties. Hemolyzed and icteric samples are not suitable for use in this assay.*

Some MMPs may be released upon platelet activation. For example, to measure circulating levels of MMP-9, platelet-poor plasma should be used. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical and Laboratory Standards Institute (CLSI), result in incomplete removal of platelets or platelet activation. This may cause variable and irreproducible results for assays of factors contained in platelets and released by platelet activation.

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Use polypropylene tubes.

Note: *On the day of the assay, ALL fresh and previously frozen serum and plasma samples require centrifugation at 16,000 x g for 4 minutes immediately prior to use or dilution.*

Analyte Incompatibilities:
When assaying serum and plasma samples, EMMPRIN cannot be multiplexed with MMP-7, MMP-8, MMP-10, MMP-12, or MMP-13 (R&D Systems®, Catalog #'s LMPM907, LMPM908, LMPM910, LMPM919, and LMPM511, respectively).

Human MMP-3 (R&D Systems®, Catalog # LMPM513) cannot be multiplexed with human MMP-10 (R&D Systems®, Catalog # LMPM910).

Cell culture supernate, serum, plasma, and platelet-poor plasma samples require a 5-fold dilution. A suggested 5-fold dilution is 40 µL of sample + 160 µL of Calibrator Diluent RD5-37. Mix thoroughly.

MMP-2, MMP-8, MMP-9, and MMP-12 serum and plasma samples must be further diluted 10-fold to a final 50-fold dilution. A suggested 50-fold dilution is 20 µL of the 5-fold diluted sample + 180 µL of Calibrator Diluent RD5-37. Mix thoroughly.

Saliva samples require a 40-fold dilution. A suggested 40-fold dilution is 10 µL of sample + 390 µL of Calibrator Diluent RD5-37. Mix thoroughly.

Urine samples require a 10-fold dilution. A suggested 10-fold dilution is 15 µL of sample + 135 µL of Calibrator Diluent RD5-37. Mix thoroughly.

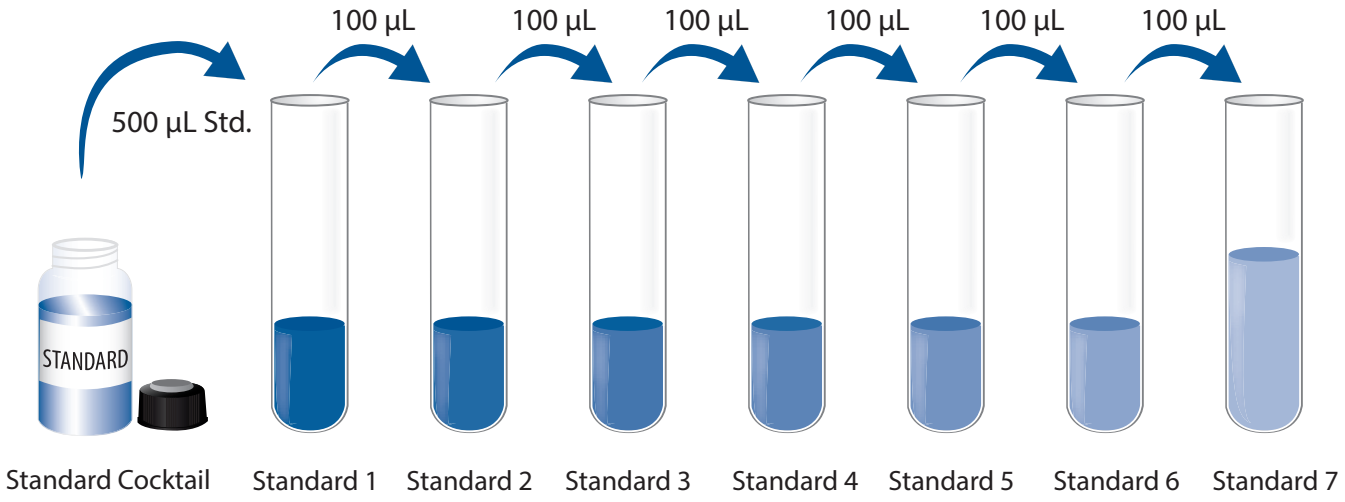
REAGENT PREPARATION

Bring all reagents to room temperature before use.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

Standard - **Refer to the Standard Value Card for the reconstitution volume and assigned values.** Reconstitute the Standard Cocktail with Calibrator Diluent RD5-37. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 500 µL of reconstituted Standard into a tube labeled Standard 1. Pipette 200 µL of Calibrator Diluent RD5-37 into the remaining tubes. Use Standard 1 to produce a 3-fold dilution series (below). Mix each tube thoroughly before the next transfer. Standard 1 serves as the high standard. Calibrator Diluent RD5-37 serves as the blank.



DILUTED MICROPARTICLE COCKTAIL PREPARATION

1. Centrifuge the Microparticle Cocktail vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial to resuspend the microparticles, taking precautions not to invert the vial.
3. Dilute the Microparticle Cocktail in the mixing bottle provided.

Number of Wells Used	Microparticle Cocktail	+	Microparticle Diluent 3
96	1000 µL	+	4.5 mL
72	750 µL	+	3.375 mL
48	500 µL	+	2.25 mL
24	250 µL	+	1.125 mL

Note: *Protect microparticles from light during handling. Diluted microparticles cannot be stored. Prepare microparticles within 30 minutes of use.*

DILUTED BIOTIN-ANTIBODY COCKTAIL PREPARATION

1. Centrifuge the Biotin-Antibody Cocktail vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Dilute the Biotin-Antibody Cocktail in Biotin Antibody Diluent 2. Mix gently.

Number of Wells Used	Biotin-Antibody Cocktail	+	Biotin Antibody Diluent 2
96	1000 µL	+	4.5 mL
72	750 µL	+	3.375 mL
48	500 µL	+	2.25 mL
24	250 µL	+	1.125 mL

STREPTAVIDIN-PE PREPARATION

Use a polypropylene amber bottle or a polypropylene tube wrapped with aluminum foil. Protect Streptavidin-PE from light during handling and storage.

1. Centrifuge the Streptavidin-PE vial for 30 seconds at 1000 x g prior to removing the cap.
2. Gently vortex the vial, taking precautions not to invert the vial.
3. Dilute the Streptavidin-PE concentrate in Wash Buffer.

Number of Wells Used	Streptavidin-PE Concentrate	+	Wash Buffer
96	55.0 µL	+	5.50 mL
72	42.0 µL	+	4.10 mL
48	28.0 µL	+	2.75 mL
24	14.0 µL	+	1.35 mL

INSTRUMENT SETTINGS

Note: *Adjust the probe height setting on the analyzer to avoid puncturing the plate. Calibrate the analyzer using the proper reagents for superparamagnetic microparticles (refer to instrument manual).*

Luminex® MAGPIX® analyzer:

a) Sample volume: 50 µL

b) Assign the microparticle region for each analyte being measured (see page 1)

c) 50 count/region

d) Collect Median Fluorescence Intensity (MFI)

Luminex® 100/200™, Luminex® FLEXMAP 3D® and Bio-Rad Bio-Plex analyzers:

Note: *Ensure that the instrument flow rate is set to the default of 60 µL/minute (fast) for all flow based analyzers.*

a) Sample volume: 50 µL

b) Bead Type:

i. Luminex® 100/200™ and FLEXMAP 3D® select MagPlex

ii. Bio-Rad Bio-Plex Manager use Bio-Plex MagPlex Beads (Magnetic)

c) Doublet Discriminator gates:

i. Luminex® 100/200™ and FLEXMAP 3D® set at 8000 and 16,500

ii. Bio-Rad Bio-Plex Manager set at 8000 and 23,000

d) Reporter Gain Setting:

i. Luminex® 100/200™ use Default setting

ii. Luminex® FLEXMAP 3D® use Enhanced PMT (High) setting

iii. Bio-Rad Bio-Plex Manager use the low RP1 target value for the CAL2 setting

e) Assign the microparticle region for each analyte being measured (see page 1)

f) 50 count/region

g) Collect MFI

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, controls, and samples be assayed in duplicate.

Note: *Protect microparticles and Streptavidin-PE from light at all times.*
MMPs are detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Add 50 µL of standard, control, or sample* per well. A plate layout is provided to record standards and samples assayed.
3. Resuspend the diluted Microparticle Cocktail by inversion or vortexing. Add 50 µL of the Microparticle Cocktail to each well of the microplate. Securely cover with a foil plate sealer. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 800 ± 50 rpm.
4. Using a magnetic device designed to accommodate a microplate, wash by applying the magnet to the bottom of the microplate, allow 1 minute before removing the liquid, filling each well with Wash Buffer (100 µL) and allow 1 minute before removing the liquid again. Complete removal of liquid is essential for good performance. **Note:** ***Do NOT blot; this may cause a loss of microparticles.*** Perform the wash procedure three times.

   Note: *Refer to the magnetic device user manual for proper wash technique using a round bottom microplate.*
5. Add 50 µL of diluted Biotin-Antibody Cocktail to all wells. Securely cover with a foil plate sealer and incubate for 1 hour at room temperature on the shaker set at 800 ± 50 rpm.
6. Repeat the wash as in step 4.
7. Add 50 µL of diluted Streptavidin-PE to all wells. Securely cover with a foil plate sealer and incubate for 30 minutes at room temperature on the shaker set at 800 ± 50 rpm.
8. Repeat the wash as in step 4.
9. Resuspend the microparticles by adding 100 µL of Wash Buffer to each well. Incubate for 2 minutes on the shaker set at 800 ± 50 rpm.
10. Read within 90 minutes using the Luminex® or Bio-Rad analyzer.
    Note: *Resuspend microparticles immediately prior to reading by shaking the plate for 2 minutes on the plate shaker at 800 ± 50 rpm.*

*Samples require dilution. See Sample Preparation section.

ASSAY PROCEDURE SUMMARY

Note: *Protect microparticles and Streptavidin-PE from light at all times.*

❶ Prepare all reagents as instructed.

↓

❷ Add 50 µL of standard, control, or sample* to each well.

↓

❸ Add 50 µL of diluted Microparticle Cocktail to each well.
Incubate for 2 hours at RT on a shaker at 800 rpm.

↓

❹ Wash by removing the liquid from each well, filling with 100 µL Wash Buffer, and removing the liquid again.
Perform the wash 3 times.

↓

❺ Add 50 µL of diluted Biotin-Antibody Cocktail to each well.
Cover and incubate for 1 hour at RT on the shaker at 800 rpm.

↓

❻ Repeat the wash as in step 4.

↓

❼ Add 50 µL of diluted Streptavidin-PE to each well.
Incubate for 30 minutes at RT on the shaker at 800 rpm.

↓

❽ Repeat the wash as in step 4.

↓

❾ Add 100 µL of Wash Buffer to each well.
Incubate for 2 minutes at RT on the shaker at 800 rpm.

↓

❿ Read within 90 minutes using a Luminex® or Bio-Rad analyzer
Note: *Resuspend microparticles immediately prior to reading.*

*Samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Use the Standard concentrations on the Standard Value Card and calculate 3-fold dilutions for the remaining levels. Average the duplicate readings for each standard and sample and subtract the average blank Median Fluorescence Intensity (MFI).

Create a standard curve for each analyte by reducing the data using computer software capable of generating a five parameter logistic (5-PL) curve-fit.

Since samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

CALIBRATION

This assay is calibrated against highly purified recombinant human MMPs produced at R&D Systems®.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.