MSD® MULTI-SPOT Assay System

Human MMP 3-Plex Ultra-Sensitive Kit

1-Plate Kit	K15034C-1
5-Plate Kit	K15034C-2
25-Plate Kit	K15034C-4

MSD Biomarker Assays

Human MMP 3-Plex Ultra-Sensitive Kit

MMP-1, MMP-3, MMP-9

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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Ordering Information

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Introduction

Matrix metalloproteinases (MMP) are a family of endopeptidases which are critically important in extracellular matrix remodeling. They are highly homologous to each other and belong to a larger family of proteases known as the metzincin superfamily. The MMP family currently includes more than 25 members that can be divided into collagenases, gelatinases, stromelysins, matrilysins and the membrane-type MMPs. The MMPs are important for several normal functions such as embryonic development, wound healing, etc. They have also been implicated in many pathological conditions such as angiogenesis, inflammation, respiratory, cardiovascular and central nervous diseases.

MMP-1 cleaves collagens of types I, II, III, VII and X. It is thought to play an important role in the pathogenesis of rheumatoid arthritis and osteoarthritis by mediating cartilage and bone destruction. It has been found to be up-regulated in a variety of cancers and has been suggested to be associated with tumor invasion and metastasis. In addition to collagen, MMP-1 also cleaves a number of non-matrix substrates and probably plays a role in the regulation of cellular behavior.

MMP-3, also called Stromelysin-1 plays an important role in the degradation and reconstitution of extracellular matrix. MMP-3 is produced as an inactive proform and is proteolytically cleaved to its activated form. An important function of MMP-3 is to activate other latent-type MMPs, such as MMP-9, which are originally secreted as proenzymes. Recent studies have implicated MMP-3 in the pathogenesis of Alzheimer's disease. It has also been associated with conditions such as rheumatoid arthritis and cancer cell invasion.

MMP-9 is a glycoprotein that plays a significant role in tissue remodeling, morphogenesis and cytokine activation. It degrades interstitial collagens, proteoglycan core protein, elastin and type IV collagen in the basement membrane. MMP-9 can also cleave several non-matrix molecules such as IL-1β, IL-8, platelet factor-4, GROα, and amyloid β peptide. It is thought to play an important role in various disease processes, including cancer, rheumatoid arthritis and atherosclerosis.

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Principle of the Assay

MSD assays provide a rapid and convenient method for measuring the levels of protein targets within a single small-volume sample. The assays are available in both singleplex and multiplex formats. In a singleplex assay, an antibody for a specific protein target is coated on one electrode (or "spot") per well. In a multiplex assay, an array of capture antibodies against different targets is patterned on distinct spots in the same well. The Human MMP 3-Plex Assay detects MMP-1, MMP-3, and MMP-9 in a sandwich immunoassay format (Figure 1). MSD provides a plate that has been pre-coated with capture antibody on spatially distinct spots – antibodies for MMP-1, MMP-3, and MMP-9. The user adds the sample and a solution containing the labeled detection antibodies—anti-MMP-1, anti-MMP-3, and anti-MMP-9 labeled with an electrochemiluminescent compound, MSD SULFO-TAG™ label—over the course of one or more incubation periods. Analytes in the sample bind to capture antibodies immobilized on the working electrode surface; recruitment of the labeled detection antibodies by bound analytes completes the sandwich. The user adds an MSD read buffer that provides the appropriate chemical environment for electrochemiluminescence and loads the plate into an MSD SECTOR® instrument for analysis. Inside the SECTOR instrument, a voltage applied to the plate electrodes causes the labels bound to the electrode surface to emit light. The instrument measures intensity of emitted light to afford a quantitative measure of MMP-1, MMP-3, and MMP-9 present in the sample.

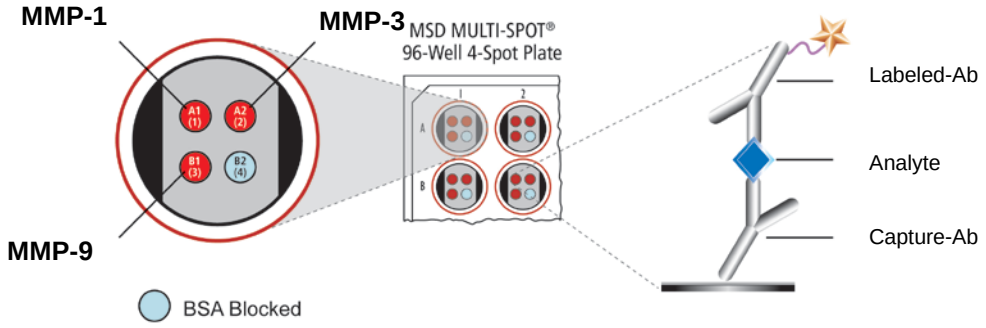


Figure 1. *Spot diagram showing placement of analyte capture antibody. The numbering convention for the different spots is maintained in the software visualization tools, on the plate packaging, and in the data files. A unique bar code label on each plate allows complete traceability back to MSD manufacturing records.*

Reagents Supplied

Product Description	Storage	Quantity per Kit K15034C-1	K15034C-2	K15034C-4
MULTI-SPOT® 96-well 4-Spot Human MMP 3-Plex Plate N45034A-1	2–8°C	1 plate	5 plates	25 plates
SULFO-TAG Detection Antibody Blend[1] (50X)	2–8°C	1 vial (75 µL)	1 vial (375 µL)	5 vials (375 µL ea)
Human MMP 3-Plex Calibrator Blend (1 µg/mL of MMP-1 and MMP-3, 5 µg/mL of MMP-9)	≤-70°C	1 vial (20 µL)	5 vials (20 µL ea)	25 vials (20 µL ea)
Diluent 2 R51BB-4 (8 mL) R51BB-3 (40 mL)	≤-10°C	2 bottles (8 mL ea)	2 bottles (40 mL ea)	6 bottles (40 mL ea)
Diluent 3 R51BA-4 (5 mL) R51BA-5 (25 mL)	≤-10°C	1 bottle (5 mL)	1 bottle (25 mL)	5 bottles (25 mL ea)
Read Buffer T (4X) R92TC-3 (50 mL) R92TC-2 (200 mL)	RT	1 bottle (50 mL)	1 bottle (50 mL)	2 bottles (200 mL ea)

Required Materials and Equipment - not supplied

- Deionized water for diluting concentrated buffers
- 50 mL tubes for reagent preparation
- 15 mL tubes for reagent preparation
- Microcentrifuge tubes for preparing serial dilutions
- Phosphate buffered saline plus 0.05% Tween-20 (PBS-T) for plate washing
- Appropriate liquid handling equipment for desired throughput, capable of dispensing 10 to 150 µL into a 96-well microtiter plate
- Plate washing equipment: automated plate washer or multichannel pipette
- Adhesive plate seals
- Microtiter plate shaker

Safety

Safe laboratory practices and personal protective equipment such as gloves, safety glasses, and lab coats should be used at all times during the handling of all kit components. All hazardous samples should be handled and disposed of properly, in accordance with local, state, and federal guidelines.

[1] SULFO-TAG conjugated detection antibodies should be stored in the dark.

Reagent Preparation

Bring all reagents to room temperature and thaw the Calibrator stock on ice.

> **Important:** Upon first thaw, separate Diluent 2 and Diluent 3 into aliquots appropriate to the size of your assay needs. These diluents can go through up to three freeze-thaw cycles without significantly affecting the performance of the assay.

Prepare Calibrator and Control Solutions

Calibrator for the Human MMP 3-Plex Assay is supplied at 10-fold higher concentration than the recommended highest Calibrator. Prepare the highest Calibrator point by diluting the stock Calibrator 10-fold in Diluent 2. MSD recommends the preparation of an 8-point standard curve consisting of at least 2 replicates of each point. Each well requires 25 µL of Calibrator. For the assay, MSD recommends 4-fold serial dilution steps and Diluent 2 alone for the 8th point:

	Human MMP 3-Plex Calibrator Blend (pg/mL)			
Standard	**MMP-1**	**MMP-3**	**MMP-9**	**Dilution Factor**
10X Stock	1000000	1000000	5000000	
STD-01	100000	100000	500000	10
STD-02	25000	25000	125000	4
STD-03	6250	6250	31250	4
STD-04	1563	1563	7813	4
STD-05	391	391	1953	4
STD-06	98	98	488	4
STD-07	24	24	122	4
STD-08	0	0	0	n/a

To prepare this 8-point standard curve for up to 4 replicates:

1) Prepare the highest Calibrator point (STD-01) by transferring 15 µL of the Human MMP 3-Plex Calibrator Blend to 135 µL Diluent 2.
2) Prepare the STD-02 by transferring 40 µL of the Human MMP 3-Plex diluted stock Calibrator to 120 µL Diluent 2. Repeat 4-fold serial dilutions 5 additional times to generate 7 Calibrators.
3) The recommended 8th Standard is Diluent 2 (i.e. zero Calibrator).

Notes:

a. Alternatively, Calibrators can be prepared in the sample matrix or diluent of choice to verify acceptable performance in these matrices. In general, the presence of some protein (for example, 1% BSA) in the sample matrix is helpful for preventing loss of analyte by adsorption onto the sides of tubes, pipette tips, and other surfaces. If your sample matrix is serum-free tissue culture media, then the addition of 10% FBS or 1% BSA is recommended.

b. The standard curve can be modified as necessary to meet specific assay requirements.

Dilution of Samples

Serum and Plasma

All solid material should be removed by centrifugation. Plasma prepared in heparin tubes commonly displays additional clotting following the thawing of the sample. Remove any additional clotted material by centrifugation. Avoid multiple freeze/thaw cycles for serum and plasma samples. Some analytes in this matrix are extremely sensitive to multiple freeze/thaw cycles and the ability to detect these analytes may decrease following the first round of thawing. *Serum and plasma samples should be diluted 10-fold into Diluent 2 prior to being used in the MSD Human MMP 3-plex Assay.* Serum or plasma with higher-than-normal levels of MMP may require a higher dilution ratio. EDTA and citrate as anticoagulants samples are not recommended due to their chelating properties, since activity of MMPs requires zinc and calcium.

Tissue Culture

Tissue culture supernatant samples may not require dilution prior to being used in the MSD Human MMP 3-Plex Assay. If using serum-free medium, the presence of carrier protein (e.g., 1% BSA) in the solution is helpful to prevent loss of analyte to the labware. Samples from experimental conditions with extremely high levels of cytokines may require a dilution.

Other Matrices

Information on preparing samples in other matrices, including sputum, CSF, and tissue homogenates can be obtained by contacting MSD Scientific Support at 1-301-947-2025 or ScientificSupport@mesoscale.com.

Prepare Detection Antibody Solution

The Detection Antibody Blend is provided at 50X stock solution. The final concentration of the working Detection Antibody Solution should be at 1X. For each plate used, dilute a 60 µL aliquot of the stock Detection Antibody Blend into 2.94 mL of Diluent 3.

Prepare Read Buffer

The Read Buffer should be diluted 2-fold in deionized water to make a final concentration of 2X Read Buffer T. Add 10 mL of 4X Read Buffer T to 10 mL of deionized water for each plate.

Prepare MSD Plate

This plate has been pre-coated with antibody for the analyte shown in Figure 1. The plate can be used as delivered; no additional preparation (e.g., pre-wetting) is required. The plate has also been exposed to a proprietary stabilizing treatment to ensure the integrity and stability of the immobilized antibodies.

Assay Protocol

Notes

1. **Addition of Diluent 2:** Dispense 25 µL of Diluent 2 into each well. Seal the plate with an adhesive plate seal and incubate for 30 min with vigorous shaking (300–1000 rpm) at room temperature.
2. **Addition of the Sample or Calibrator:** Dispense 25 µL of sample or Calibrator into separate wells of the MSD plate. Seal the plate with an adhesive plate seal and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.

Shaking a 96-well MSD plate typically accelerates capture at the working electrode.

3. **Wash and Addition of the Detection Antibody Solution:** Wash the plate 3 times with PBS-T. Dispense 25 µL of the 1X Detection Antibody Solution into each well of the MSD plate. Seal the plate and incubate for 2 hours with vigorous shaking (300–1000 rpm) at room temperature.
4. **Wash and Read:** Wash the plate 3 times with PBS-T. Add 150 µL of 2X Read Buffer T to each well of the MSD plate. Analyze the plate on the SECTOR Imager. Plates may be read immediately after the addition of Read Buffer.

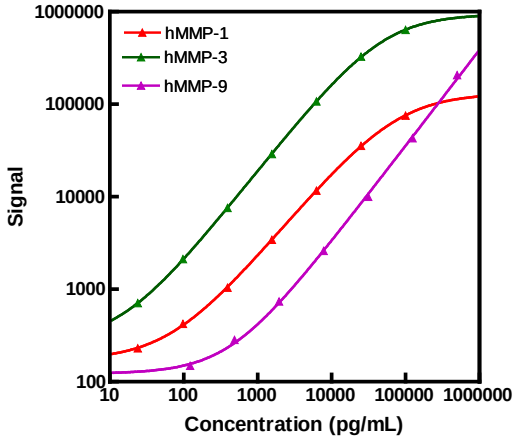
Bubbles in the fluid will interfere with reliable reading of plate. Use reverse pipetting techniques to insure bubbles are not created when dispensing the Read Buffer.

Analysis of Results

The Calibrators should be run in duplicate to generate a standard curve. The standard curve is modeled using least squares fitting algorithms so that signals from samples with known levels of the analyte of interest can be used to calculate the concentration of analyte in the sample. The assays have a wide dynamic range (3–4 logs) which allows accurate quantitation in many samples without the need for dilution. The MSD DISCOVERY WORKBENCH® analysis software utilizes a 4-parameter logistic model (or sigmoidal dose-response) and includes a $1/Y^2$ weighting function. The weighting function is important because it provides a better fit of data over a wide dynamic range, particularly at the low end of the standard curve.

Typical Standard Curve

The following standard curves are an example of the dynamic range of the assay. The actual signals may vary and a standard curve should be run for each set of samples and on each plate for the best quantitation of unknown samples.



MMP-1		
Conc. (pg/mL)	Average Signal	%CV
0	159	4.9
24	230	4.9
98	422	5.4
391	1041	3.7
1563	3431	3.6
6250	11645	2.3
25000	35539	4.6
100000	75387	2.7

MMP-3		
Conc. (pg/mL)	Average Signal	%CV
0	179	3.7
24	707	1.0
98	2117	2.5
391	7578	1.4
1563	28879	5.3
6250	107273	3.8
25000	327025	5.5
100000	634272	3.7

MMP-9		
Conc. (pg/mL)	Average Signal	%CV
0	117	3.9
122	149	1.2
488	284	2.3
1953	739	1.4
7813	2593	1.0
31250	9994	4.8
125000	42839	2.2
500000	205905	1.5

Sensitivity

The lower limit of detection (LLOD) is the calculated concentration of the signal that is 2.5 standard deviations over the zero Calibrator. The values below represent the average LLOD over multiple kit lots.

	MMP-1	MMP-3	MMP-9
LLOD (pg/mL)	11	2.1	99

Specificity

To determine the specificity of the assays, MMP Calibrators were assayed individually in Diluent 2 with MMP 3-plex plates. The MMP-1 Calibrator displayed 0.6% cross-reactivity with the MMP-9 spot, and the MMP-3 Calibrator displayed 0.4% and 1.5% cross-reactivity with the MMP-1 and MMP-9 spots respectively. Recombinant human MMP-2, MMP-7, MMP-10, TIMP-1 and TIMP-2 proteins were also tested. MMP-10 Calibrator presented 8.4% cross-reactivity to the MMP-3 spot. Less than 0.3% cross-reactivity was observed for MMP-1, 3 and 9 spots with MMP-2, MMP-7, TIMP-1 and TIMP-2 proteins. While the TIMP1 and TIMP2 proteins do not show detectable signal when assayed individually, we have found that TIMP-1 affects measurements of MMP-9 in serum by lowering counts values in a dose-dependent way.

Assay Components

The human MMP-1, MMP-3, and MMP-9 capture and detection antibodies used in this assay are listed below.

	Source species	
Analyte	**MSD Capture Antibody**	**MSD Detection Antibody**
MMP-1	Goat Polyclonal	Goat Polyclonal
MMP-3	Goat Polyclonal	Goat Polyclonal
MMP-9	Mouse Monoclonal	Goat Polyclonal

Summary Protocol

MSD 96-well MULTI-SPOT Human MMP 3-Plex Ultra-Sensitive Kit

MSD provides this summary protocol for your convenience.
Please read the entire detailed protocol prior to performing the
MSD Human MMP 3-Plex Assay.

Sample and Reagent Preparation

Bring all reagents to room temperature and thaw the Calibrator stock on ice.

Serum and plasma samples should be diluted 10-fold in Diluent 2.

Prepare Calibrator solutions and standard curve.

Use the 10X Calibrator stock to prepare an 8-point standard curve by diluting in Diluent 2.

Note: *The standard curve can be modified as necessary to meet specific assay requirements.*

Prepare Detection Antibody Solution by diluting Detection Antibody Blend to 1X in a final volume of 3.0 mL Diluent 3 per plate.

Prepare 20 mL of 2X Read Buffer T by diluting 4X Read Buffer T with deionized water.

SERUM OR PLASMA SAMPLES

Step 1: Add Diluent 2

Dispense 25 µL/well Diluent 2.

Incubate at room temperature with vigorous shaking (300-1000 rpm) for 30 minutes.

Step 2: Add Sample or Calibrator

Dispense 25 µL/well Calibrator or sample.

Incubate at room temperature with vigorous shaking (300-1000 rpm) for 2 hours.

Step 3: Wash and Add Detection Antibody Solution

Wash plate 3 times with PBS-T.

Dispense 25 µL/well 1X Detection Antibody Solution.

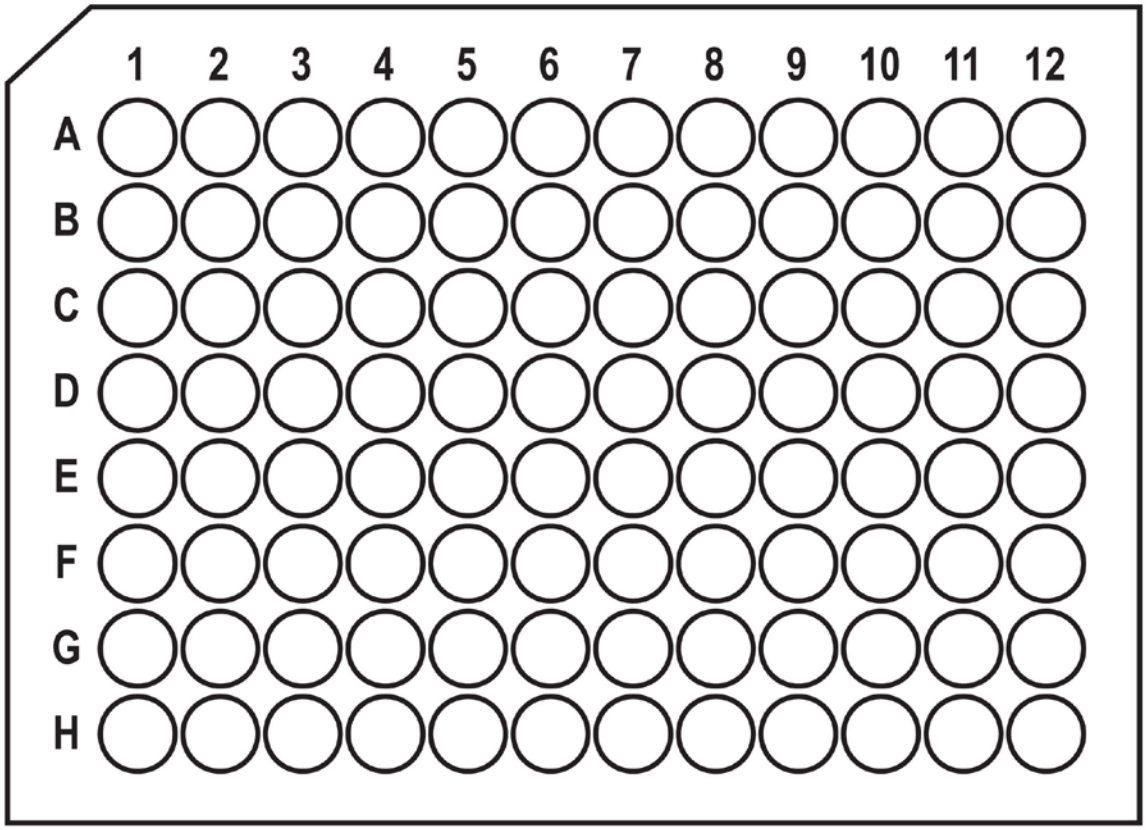
Incubate at room temperature with vigorous shaking (300-1000 rpm) for 2 hours.

Step 4: Wash and Read Plate

Wash plate 3 times with PBS-T.

Dispense 150 µL/well 2X Read Buffer T.

Analyze plate on SECTOR Imager instrument.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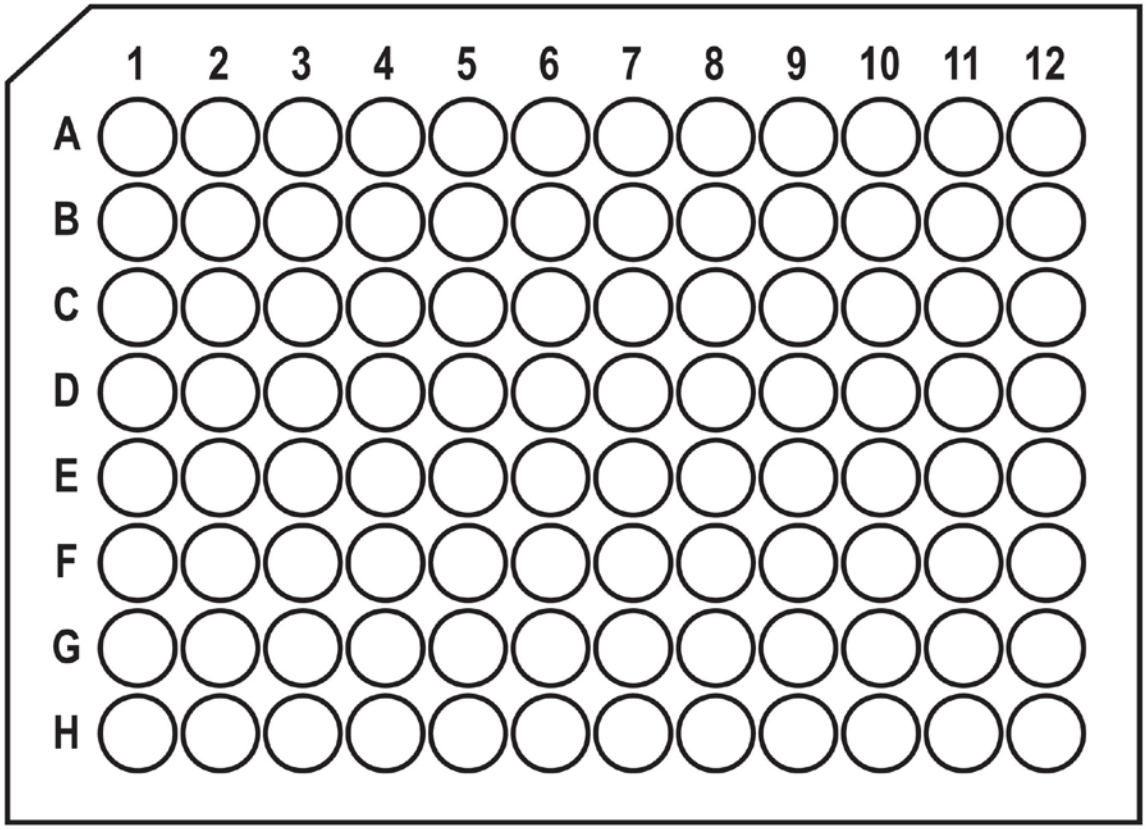
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