Fluorokine® E

Human Active MMP-9 Fluorescent Assay

Catalog Number F9M00

For the quantitative determination of human active Matrix Metalloproteinase 9 (MMP-9) in cell culture supernates, serum, plasma, 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

Matrix metalloproteinases (MMPs), also called matrixins, constitute a family of zinc and calcium dependent endopeptidases that function in the breakdown of extracellular matrix (ECM). They play an important role in many normal physiological processes such as embryonic development, morphogenesis, reproduction and tissue remodeling (1). They also participate in many pathological processes such as arthritis, cancer and cardiovascular disease (2). While the amounts of newly synthesized MMPs are regulated mainly at the levels of transcription, the proteolytic activities of existing MMPs are controlled through both the activation of pro-enzymes or zymogens and the inhibition of active enzymes by endogenous inhibitors, α_2-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs).

MMP-9 (also referred to as gelatinase B, 92 kDa type IV collagenase, 92 kDa gelatinase, and type V collagenase) is secreted as a 92 kDa glycosylated pro-enzyme (3). Activation of the pro-enzyme involves a proteolytic removal of the N-terminal pro-region containing the cysteine switch motif conserved in MMPs (4). The resulting 82 kDa active enzyme consists of a catalytic domain with a zinc-binding motif conserved in metzincins (5, 6). The catalytic domain also contains three contiguous fibronectin type II homology units responsible for binding gelatin (7). A proline-rich hinge region links the catalytic domain to the C-terminal hemopexin-like domain. *In vitro* treatment of the pro-enzyme with 4-aminophenylmercuric acetate (APMA) produces not only the 82 kDa active enzyme but also a C-terminal truncated form of approximately 65 kDa with the activity comparable to that of the 82 kDa form (8).

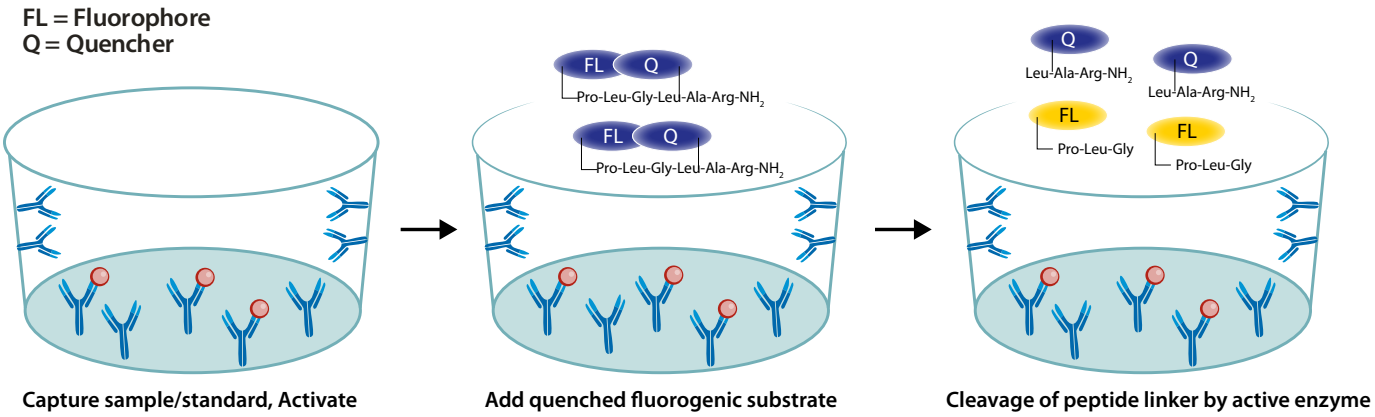
MMP-9 degrades components of the ECM with high specific activity for denatured collagens (gelatin). It can cleave native collagens of type IV, V, and XI, and elastin. MMP-9 can also cleave a variety of non-ECM molecules such as interleukin (IL)-1β, IL-8, connective tissue-activating peptide-III, platelet factor-4, GROα, substance P, myelin basic protein, and amyloid β peptide. MMP-9 can increase or decrease the biological activity of these molecules, depending upon the site of cleavage (9, 10).

MMP-9 is produced by a variety of normal and transformed cells including neutrophils, monocytes, macrophages, fibroblasts, osteoclasts, chondrocytes, keratinocytes, endothelial and epithelial cells and regulated by various agents. Mouse models deficient in MMP-9 expression have shown that it is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes (11), suppresses development of experimental abdominal aortic aneurysms (12), is required for blister formation (13), contributes to skin carcinogenesis (14), and inactivates the serpin α1-proteinase inhibitor (15).

The Human Active MMP-9 Fluorokine E kit combines the specificity of a monoclonal antibody that captures all three MMP-9 forms (92, 82, and 65 kDa) but not other MMPs and the sensitivity of fluorescence. The kit is designed to measure the levels of endogenous active MMP-9 in cell culture supernates, serum, plasma, and urine and the MMP-9 in these samples that can be activated by APMA during the assay procedure. The determined MMP-9 activity may reflect the balance of MMP-9 and its inhibitor TIMPs. Therefore, it is recommended that the levels of TIMPs be determined in order to interpret the results properly. For example, TIMP-1 inhibits the activity of MMP-9 through binding of the N-terminal domain of the TIMP-1 to the active site of MMP-9. In addition, TIMP-1 also binds to pro-MMP-9 through the interaction between the C-terminal domains of both proteins (16).

PRINCIPLE OF THE ASSAY

This assay is a fluorometric assay designed to quantitatively measure enzyme activity. A monoclonal antibody specific for human MMP-9 has been pre-coated onto a black microplate. Standards and samples are pipetted into the wells and any MMP-9 is bound by the immobilized antibody. After washing away any unbound substances, an activation reagent (APMA) is added to standards and selected samples*. Following a wash, a fluorogenic substrate linked to a quencher molecule is added and any active enzyme present will cleave the peptide linker between the fluorophore and the quencher molecule. This cleavage eliminates the distance dependent resonance energy transfer between the fluorophore and the quencher molecule, allowing a fluorescent signal that is proportional to the amount of enzyme activity in the sample.



*The kit is designed to measure the levels of both endogenous active MMP-9 in serum, plasma, urine and cell culture supernates and the MMP-9 in these samples that can be activated by APMA during the assay procedure.

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 generate values higher than the highest standard, further dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in standard 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 all factors have been tested in the Fluorokine E Immunoassay, the possibility of interference cannot be excluded.
- Relative fluorescence units (RFU) may differ among fluorimeters. The Human Active MMP-9 Fluorokine E Assay was optimized using a Molecular Devices fMax™ fluorimeter. Other instruments may require settings to be adjusted.

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.
- A humidified environment can be made by placing moist paper towels in a sealed container.

PRECAUTIONS

Some components of this kit contain sodium azide which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

The APMA provided with this kit is a mercury containing compound. The total amount of mercury in this kit is 20 mg. Dispose of according to local, state, and federal regulations.

The APMA and Substrate provided with this kit are hazardous components containing DMSO. Wear gloves and protective clothing when handling these materials. Dispose of according to local, state, and federal regulations.

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

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

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/ RECONSTITUTED MATERIAL
Human MMP-9 Microplate	890837	96 well black polystyrene microplate (6 strips of 16 wells) coated with monoclonal antibody specific for human MMP-9.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Active MMP-9 Standard	890838	3 vials of recombinant human pro-MMP-9 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a fresh standard for each assay.
Assay Diluent RD1X	895121	11 mL of a buffered protein base with preservatives. *May contain crystals. Warm to room temperature, and mix well before and during use. For urine samples.*	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1N	895488	12 mL of a buffered protein base with preservatives. *For cell culture supernates/serum/plasma samples.*	
Calibrator Diluent RD5-23	895288	21 mL of a buffered protein base with preservatives. *For urine samples.*	
Calibrator Diluent RD5-24 Concentrate	895325	21 mL of a concentrated buffered protein base with preservatives. *For cell culture supernate/serum/ plasma samples. Use diluted 1:5 in this assay.*	
Reagent Diluent 1	895289	2 vials (22.5 mL/vial) of a Tris-HCl buffer with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
***p*-Aminophenylmercuric Acetate (APMA)**	895327	200 µL of a stock solution of 0.5 M APMA in DMSO.	Discard after use. Prepare fresh for each assay. Store stock solutions for up to 1 month at 2-8 °C.*
Substrate	895326	300 µL of a stock solution of 1 mM fluorogenic substrate in DMSO.	
Plate Sealers	N/A	8 adhesive strips.	

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

OTHER SUPPLIES REQUIRED

- *fMax* fluorimeter set with the following parameters: excitation wavelength set to 320 nm or 340 nm and emission wavelength set to 405 nm; endpoint mode; 1 x 20 mS integration time; plate speed = 6, or the equivalent.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- 37 °C incubator.
- Humidified environment (*e.g.* sealable bag with moist paper towels or humidified chamber)
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm (R&D Systems, Catalog # T4625-Q or T4625-1CEQ).
- **Polypropylene** test tubes for dilution of standards and samples.

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.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation 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.

Note: *EDTA and citrate cannot be used as anticoagulants in this assay.* *EDTA and citrate are strong and weak metal chelators, respectively. The activity of MMPs requires zinc and calcium and is therefore inhibited by these metal chelators.*

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

SAMPLE PREPARATION

Cell culture supernate, serum and plasma samples require at least a 100-fold dilution. A suggested 100-fold dilution is 10 µL sample + 90 µL Calibrator Diluent RD5-24 (diluted 1:5)* followed by 50 µL of diluted sample + 450 µL of Calibrator Diluent RD5-24 (diluted 1:5).

Urine samples require at least a 2-fold dilution. A suggested 2-fold dilution is 250 µL of sample + 250 µL of Calibrator Diluent RD5-23.

*See Reagent Preparation section.

REAGENT PREPARATION

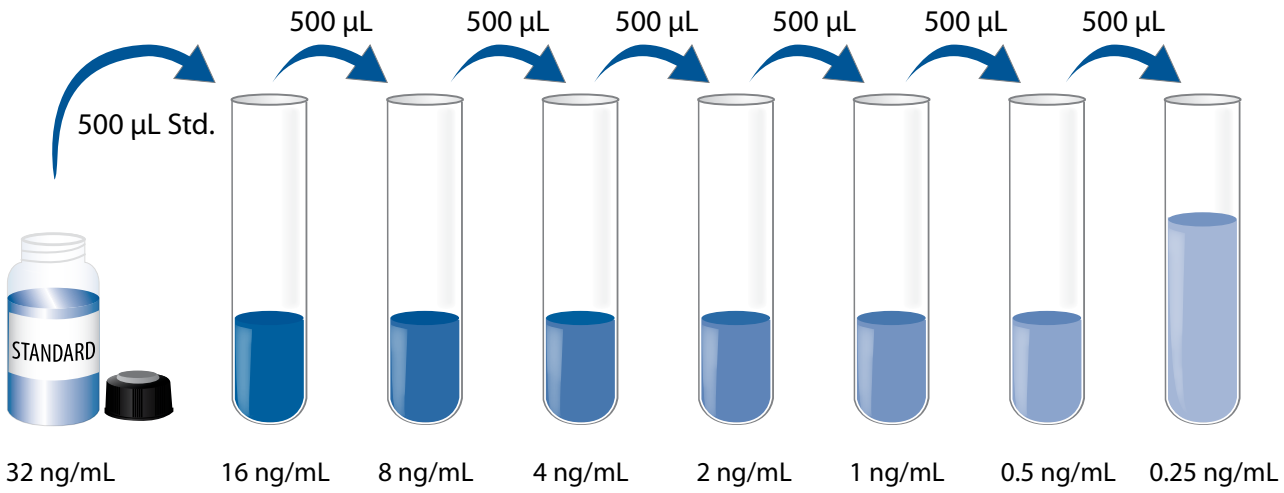
Bring all reagents to room temperature before use.
Substrate and APMA may be warmed to 37 °C.

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 deionized or distilled water to prepare 500 mL of Wash Buffer.

Calibrator Diluent RD5-24 (diluted 1:5) - Add 20 mL Calibrator Diluent RD5-24 Concentrate to 80 mL of deionized or distilled water to produce 100 mL of Calibrator Diluent RD5-24 (diluted 1:5).

Active MMP-9 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Active MMP-9 Standard with deionized or distilled water. This reconstitution produces a stock solution of 32 ng/mL. 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 Calibrator Diluent RD5-23 (*for urine samples*) or Calibrator Diluent RD5-24 (diluted 1:5) (*for cell culture supernate/serum/plasma samples*) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 16 ng/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 ng/mL).



REAGENT PREPARATION *CONTINUED*

***p*-aminophenylmercuric acetate (APMA)** - The APMA Solution should be prepared within 15 minutes of use. Tap the vial gently to dislodge any APMA in the vial cap. Prepare only the amount needed for each assay (200 µL of the diluted APMA is needed per well). Dilute APMA 168-fold with Reagent Diluent 1. Solution will appear cloudy and contain a precipitate; vortex well. Example dilutions are listed in the table below. Discard any unused diluted APMA. Prepare fresh APMA for each assay.

Note: *Prepare only the amount of APMA needed for standard wells and any desired sample wells to be activated.*

APMA Dilution

Number of Wells	APMA Stock	+	Reagent Diluent 1	Total APMA
32	42 µL	+	6.96 mL	7 mL
64	83 µL	+	13.92 mL	14 mL
96*	135 µL	+	22.5 mL	22.64 mL*

**When activating a full plate, it is recommended to spike the stock solution into the full bottle (22.5 mL) of Reagent Diluent 1. Label the bottle "APMA" to avoid reagent mixup.*

Substrate Solution - Substrate solution should be prepared within 15 minutes of use. Protect from light prior to use. Tap vial gently to dislodge any substrate from the vial cap. Prepare only the amount needed for each assay (200 µL of the diluted substrate is needed per well). Dilute Substrate stock 84-fold with Reagent Diluent 1. Example dilutions are listed in the table below. Discard any unused diluted substrate. Prepare fresh Substrate for each assay.

Substrate Dilution

Number of Wells	Substrate Stock	+	Reagent Diluent 1	Total Substrate
32	83 µL	+	6.92 mL	7 mL
64	166 µL	+	13.83 mL	14 mL
96*	270 µL	+	22.5 mL	22.77 mL*

**When assaying a full plate, it is recommended to spike the stock solution into the full bottle (22.5 mL) of Reagent Diluent 1. Label the bottle "Substrate" to avoid reagent mixup.*

ASSAY PROCEDURE

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

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. **For urine samples:** Add 50 µL of Assay Diluent RD1X to each well. *Assay Diluent RD1X may contain crystals. Warm to room temperature and mix well before and during use.*
   For cell culture supernate/serum/plasma samples: Add 50 µL of Assay Diluent RD1N to each well.
4. Add 200 µL of Standard or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 200 µL of diluted APMA to all standard wells and any desired sample wells. Cover with the adhesive strip provided. Incubate for 2 hours at 37 °C in a humidified environment. Protect from light.

   Note: *The addition of APMA will activate any potentially active forms of MMP-9 present in the sample. To measure endogenous levels of active MMP-9 in samples, do not add APMA to the sample wells. Add 200 µL Reagent Diluent 1 to these sample wells instead. APMA must always be added to the standard wells.*
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of diluted Substrate to each well. Cover with a new adhesive strip. **Protect the plate from light within 10 minutes of Substrate addition.** Incubate for 17-20 hours at **37 °C in a dark, humidified environment.**

   Note: *Exposure of Substrate to light for greater than 10 minutes may cause the Substrate to degrade. It is recommended that the addition of Substrate be performed in a low light environment, and be completed and moved to a light-free environment within 10 minutes.*
9. Determine the relative fluorescence units (RFU) of each well using a fluorescence plate reader set with the following parameters: excitation wavelength set to 320 nm or 340 nm and emission wavelength set to 405 nm; endpoint mode; 1 x 20 mS integration time; plate speed = 6.

*Samples require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

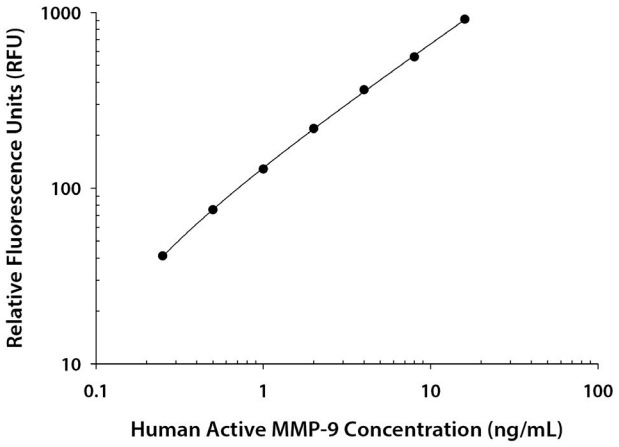
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the active MMP-9 concentrations versus the log of the RFU and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

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

TYPICAL DATA

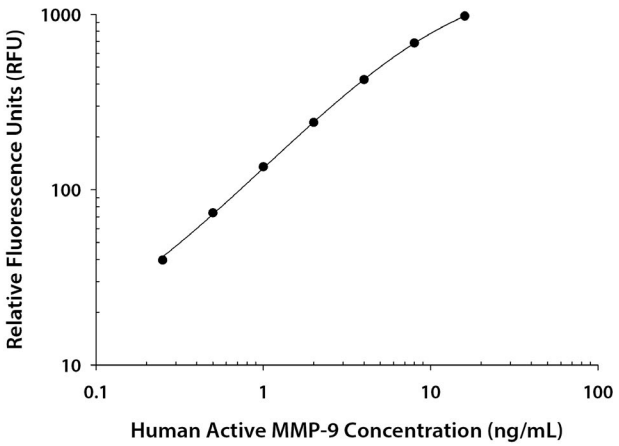
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/SERUM/PLASMA ASSAY



(ng/mL)	RFU	Average	Corrected
0	17.50 17.70	17.60	—
0.25	58.45 58.95	58.70	41.10
0.5	91.84 94.03	92.94	75.34
1	142.2 150.0	146.1	128.5
2	228.2 243.0	235.6	218.0
4	375.4 384.9	380.2	362.6
8	586.6 563.8	575.2	557.6
16	937.2 925.1	931.2	913.6

URINE ASSAY



(ng/mL)	RFU	Average	Corrected
0	19.35 18.95	19.15	—
0.25	56.52 60.90	58.71	39.56
0.5	89.88 95.78	92.83	73.68
1	155.9 152.0	154.0	134.85
2	267.4 253.6	260.5	241.4
4	454.5 432.9	443.7	424.6
8	713.7 696.4	705.0	685.8
16	978.6 1014	996.3	977.2

PRECISION

Intra-assay Precision (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in separate assays to assess inter-assay precision.

CELL CULTURE SUPERNATE/SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	22	22	22
Mean (ng/mL)	1.5	6.7	14.4	1.5	7.1	12.9
Standard deviation	0.07	0.28	0.56	0.14	0.57	1.08
CV (%)	4.8	4.2	3.9	9.3	8.0	8.4

URINE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	0.95	4.1	12.0	1.1	4.4	11.4
Standard deviation	0.06	0.26	0.41	0.06	0.29	0.83
CV (%)	6.3	6.3	3.4	5.5	6.6	7.3

RECOVERY

The recovery of active human MMP-9 spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell Culture Supernate (n=4)	100	90-110%
Urine (n=5)	98	88-108%
Serum (n=5)	100	90-110%
Heparin Plasma (n=5)	99	90-110%

SENSITIVITY

Eighty-three assays were evaluated and the minimum detectable dose (MDD) of active human MMP-9 ranged from 0.002-0.01 ng/mL. The mean MDD was 0.005 ng/mL.

The MDD was determined by adding two standard deviations to the mean RFU of twenty zero standard replicates and calculating the corresponding concentration.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of active human MMP-9 were diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay. All samples were assayed without addition of APMA.

		Cell culture supernates* (n=5)	Serum* (n=8)	Heparin plasma* (n=8)	Urine* (n=5)
1:2	Average % of Expected	103	98	98	97
	Range (%)	92-109	94-108	94-103	86-106
1:4	Average % of Expected	103	100	99	102
	Range (%)	91-112	91-108	88-108	97-108
1:8	Average % of Expected	102	104	105	100
	Range (%)	88-109	94-109	85-114	96-108
1:16	Average % of Expected	100	103	97	89
	Range (%)	87-108	98-114	89-104	87-91

Samples containing high levels of human MMP-9 were diluted with the appropriate Calibrator Diluent and assayed. Samples were activated during the assay with the addition of APMA.

		Cell culture supernates* (n=1)	Serum* (n=2)	Urine* (n=1)
1:2	Average % of Expected	100	103	101
	Range (%)	———	98-108	———
1:4	Average % of Expected	87	108	96
	Range (%)	———	101-115	———
1:8	Average % of Expected	87	112	101
	Range (%)	———	112-113	———
1:16	Average % of Expected	87	———	101
	Range (%)	———	———	———

*Samples were diluted prior to assay as directed in the Sample Preparation section.

CALIBRATION

This assay is calibrated against a highly purified CHO cell-expressed recombinant human MMP-9 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of both endogenous active human MMP-9 (no APMA) and all potentially active forms of human MMP-9 (APMA) in this assay. No medical histories were available for the donors used in this study.

MMP-9 (APMA Activated)

Sample Type	Mean of Detectable (ng/mL)	% Detectable	Range (ng/mL)
Serum (n=40)	680	98	ND-1463
Heparin Plasma (n=40)	91	100	35-221
Urine (n=32)	3.2	28	ND-46

Endogenous active MMP-9 (no APMA)

Sample Type	Mean of Detectable (ng/mL)	% Detectable	Range (ng/mL)
Serum (n=40)	91	80	ND-597
Heparin Plasma (n=40)	65	20	ND-107
Urine (n=32)	4.1	16	ND-10.9

ND=Non-detectable, < 0.25 ng/mL

Cell Culture Supernates:

Human peripheral blood lymphocytes (5 x 10^6 cells/mL) were cultured in RPMI supplemented with 5% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed and assayed for the presence of both endogenous active MMP-9 (no APMA) and all potentially active forms of human MMP-9 (APMA).

MMP-9 (APMA Activated)

Condition	Day 1 (ng/mL)	Day 5 (ng/mL)
Unstimulated	158	1705
Stimulated	58	299

Endogenous active MMP-9 (no APMA)

Condition	Day 1 (ng/mL)	Day 5 (ng/mL)
Unstimulated	66	697
Stimulated	36	144

SPECIFICITY

This assay recognizes natural and recombinant human MMP-9.

The factors listed below were prepared at 160 ng/mL in Calibrator Diluents and assayed for cross-reactivity. No significant cross-reactivity was observed.

Recombinant human:

MMP-1
MMP-2
MMP-3
MMP-8
MMP-10
MMP-12
TIMP-1
TIMP-2

Recombinant human TIMP-1 and TIMP-2 were spiked into a mid-range MMP-9 control and assayed for interference. Interference was observed with TIMP-1 at concentrations ≥ 2.5 ng/mL. Interference was observed with TIMP-2 at concentrations ≥ 80 ng/mL.

TIMP-1 Interference

TIMP-1 Concentration (ng/mL)	Observed MMP-9 Value (ng/mL)
160	3.95
80	4.66
40	5.18
20	5.50
10	6.05
5	6.47
2.5	6.57
0	7.55

TIMP-2 Interference

TIMP-2 Concentration (ng/mL)	Observed MMP-9 Value (ng/mL)
160	6.35
80	7.13
0	7.55

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