Fluorokine® E

Human Active MMP-1 Fluorescent Assay

Catalog Number F1M00

For the quantitative determination of human active Matrix Metalloproteinase 1 (MMP-1) in cell culture supernates, serum, and plasma.

This package insert must be read in its entirety before using this product.

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

TABLE OF CONTENTS

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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 proenzymes or zymogens and the inhibition of active enzymes by endogenous inhibitors, α-macroglobulins and tissue inhibitors of metalloproteinases (TIMPs).

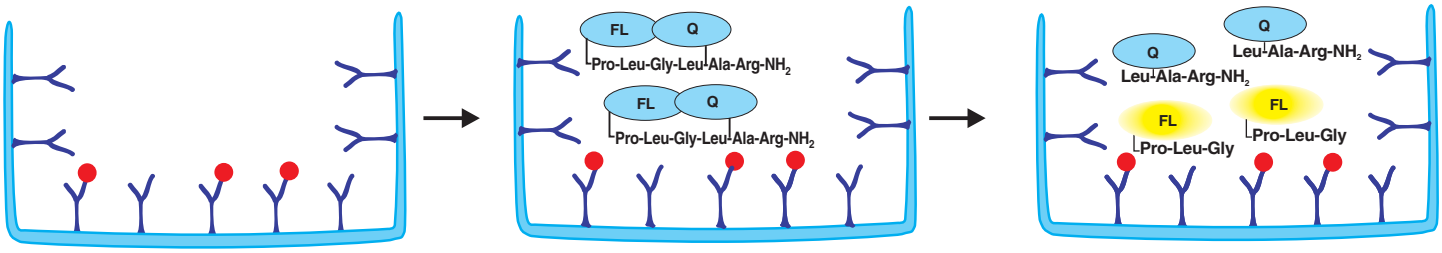
MMP-1 (also referred to as interstitial collagenase, vertebrate collagenase, fibroblast collagenase, or collagenase I) is produced by fibroblasts, chondrocytes, macrophages, keratinocytes, endothelial cells, and osteoblasts (3). The production of MMP-1 is upregulated by a variety of stimuli including cytokines such as EGF, interleukins and TNF-α, chemical agents such as cAMP and phorbol esters, and events occurring at the cell surface such as cell fusion and phagocytosis. MMP-1 is normally secreted as a 52 kDa pro-enzyme with an N-terminal pro-domain containing the cysteine switch motif conserved in MMPs (4). Activation of the pro-enzyme involves a proteolytic removal the pro-domain. The resulting 43 kDa active enzyme consists of a catalytic domain with a zinc-binding motif conserved in metzincins (5,6). A short hinge peptide links the catalytic domain to the C-terminal hemopexin-like domain. The C-terminal domain can also be removed from MMP-1 by proteinases such as MMP-3 and MMP-1 itself (3,7).

MMP-1 plays a significant role in the degradation of fibrillar collagens in the ECM remodeling, characterized by the cleavage of the interstitial collagen triple helix into ¾ and ¼ fragments. MMP-1 is therefore implicated in a wide variety of biological processes where collagen degradation occurs. These include rheumatoid arthritis, osteoarthritis, periodontal disease, tumor invasion, angiogenesis, corneal ulceration, tissue remodeling, inflammatory bowel disease, atherosclerosis, aneurysm, and restenosis. In addition, MMP-1 can also cleave a variety of other substrates such as α_2-macroglobulin, α1-proteinase inhibitor, α1-antichymotrypsin, and serum amyloid A (3). Thus, the role of MMP-1 is diverse and activation of MMP-1 is an important control point. It can be illustrated that tumor cell produced MMP-1 degrades interstitial collagens, a step essential in tumor cell invasion, but activation of MMP-1 requires a proteolytic cascade involving serine proteinases and MMP-3 contributed by stromal cells (7).

The Fluorokine E Human Active MMP-1 kit combines the specificity of a monoclonal antibody that captures both pro and active MMP-1 forms and the sensitivity of fluorescence. The kit is designed to measure the levels of both endogenous active MMP-1 in cell culture supernates, serum, and plasma and the MMP-1 in these samples that can be activated by APMA in the assay procedure. The measured MMP-1 activity may reflect the balance of MMP-1 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-1 through binding of the N-terminal domain of the TIMP-1 to the active site of MMP-1 (8). The Quantikine Human TIMP-1 Immunoassay (R&D Systems Catalog # DTM100) can be used to measure TIMP-1 concentrations in these samples.

PRINCIPLE OF THE ASSAY

This assay is a fluorimetric assay designed to quantitatively measure enzyme activity. A monoclonal antibody specific for MMP-1 has been pre-coated onto a black microplate. Standards and samples are pipetted into the wells and any MMP-1 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.



Capture sample/standard, Activate

Add quenched fluorogenic substrate

Cleavage of peptide linker by active enzyme

FL = Fluorophore
Q = Quencher

*This kit is designed to measure the levels of both endogenous active MMP-1 in serum, plasma and cell culture supernates and the MMP-1 in these samples that can be activated by APMA in 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 fall outside the dynamic range of the assay, dilute the samples with Calibrator Diluent and repeat the assay.
- Any variation in Calibrator Diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Other enzymes and proteins present in biological samples do not necessarily interfere with the measurement of active proteinases in samples. Until these factors have been tested in Fluorokine E Assay, the possibility of interference cannot be excluded.
- Relative fluorescence units (RFU) may differ among fluorimeters. The Fluorokine E Human Active MMP-1 Assay was optimized using a Molecular Devices ***f**max™ fluorimeter. Other instruments may require settings to be adjusted.*

***f**max* is a trademark of Molecular Devices Corporation.

MATERIALS PROVIDED

Active MMP-1 Microplate (Part 890689) - 96 well black polystyrene microplate (6 strips of 16 wells) coated with a monoclonal antibody against MMP-1.

MMP-1 Standard (Part 890777) - 3 vials of recombinant human pro-MMP-1 in a buffered protein base with preservative; lyophilized.

Assay Diluent RD1-64 (Part 895355) - 11 mL of a buffered protein base with preservative.

Calibrator Diluent RD5-25 (Part 895356) - 21 mL of a buffered protein base with preservative.

Reagent Diluent 2 (Part 895357) - 2 vials (22.5 mL/vial) of a Tris-HCl buffer with preservative.

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservative.

p-Aminophenylmercuric Acetate (APMA) (Part 895327) - 200 µL of a stock solution of 0.5 M APMA in DMSO.

Substrate (Part 895292) - 400 µL of a stock solution of 1 mM fluorogenic substrate in DMSO.

Plate Covers - 8 adhesive strips.

STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2 - 8° C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="8">Opened/ Reconstituted Reagents</td><td>Diluted Wash Buffer</td><td rowspan="4">May be stored for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Assay Diluent RD1-64</td></tr>
<tr><td>Calibrator Diluent RD5-25</td></tr>
<tr><td>Reagent Diluent 2</td></tr>
<tr><td>Diluted Substrate</td><td rowspan="2">Discard after use. Prepare fresh for each assay. Store stock solution for up to 1 month at 2 - 8° C.*</td></tr>
<tr><td>Diluted APMA</td></tr>
<tr><td>Standard</td><td>Discard the MMP-1 stock solution and dilutions after use. Use a fresh standard for each assay.</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*</td></tr>
</table>

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

OTHER SUPPLIES REQUIRED

- ***f**max* fluorimeter set with the following parameters: excitation wavelength set to 320 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.
- 500 mL graduated cylinder.
- 37° C incubator.
- Humidified environment (*e.g.* ziploc bag with moist paper towels or humidified chamber)
- Horizontal orbital microplate shaker (0.12” orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Test tubes for dilution.

PRECAUTIONS

The APMA and Substrate provided with this kit are hazardous components. Wear gloves and protective clothing when handling these materials.

The APMA provided with this kit is a mercury containing compound. The total amount of mercury in this kit is 20 mg.

Some components in 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.

SAMPLE COLLECTION AND STORAGE

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

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000 x g. Remove serum and assay immediately or aliquot and store 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 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.*

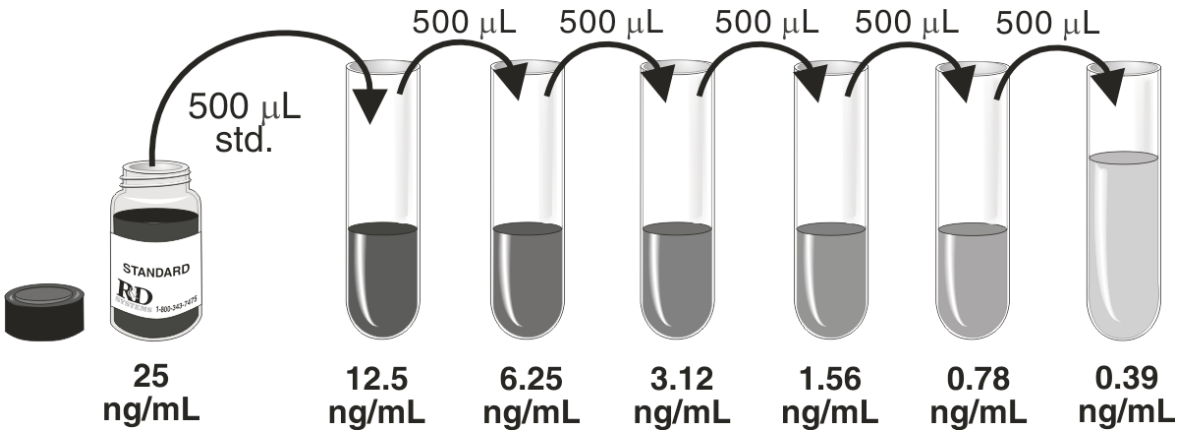
REAGENT PREPARATION

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. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

MMP-1 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the MMP-1 Standard with Calibrator Diluent RD5-25. This reconstitution produces a stock solution of 25 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 500 µL of Calibrator Diluent RD5-25 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 25 ng/mL standard serves as the high standard. The Calibrator Diluent RD5-25 serves as the zero standard (0 ng/mL).



p-aminophenylmercuric acetate (APMA) - APMA solution should be prepared within 15 minutes of use. Tap 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 1:168 with Reagent Diluent 2. 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 the APMA needed for standard wells and any desired sample wells to be activated.*

APMA Dilution					
# Wells to be activated	APMA Stock	+	Reagent Diluent 2	=	Total APMA Prepared
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 2. Label the bottle 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 vial cap. Prepare only the amount needed for each assay (200 µL of the diluted substrate is needed per well). Dilute Substrate stock 1:64 with Reagent Diluent 2. Example dilutions are listed in the table below. Discard any unused diluted substrate. Prepare fresh Substrate for each assay.

Substrate Dilution					
# Wells	Substrate Stock	+	Reagent Diluent 2	=	Total Substrate Prepared
32	109 µL	+	6.89 mL	=	7 mL
64	219 µL	+	13.78 mL	=	14 mL
96*	360 µL	+	22.5 mL	=	22.86 mL*

**When assaying a full plate, it is recommended to spike the stock solution into the full bottle (22.5 mL) of Reagent Diluent 2. Label the bottle to avoid reagent mixup.*

ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, 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. Add 100 µL of Assay Diluent RD1-64 to each well.

4. Add 150 µL of Standard, sample, or control per well. Cover with the adhesive strip provided. Incubate for 3 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record standards and samples assayed.

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 toweling.

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-1 present in the sample. To measure endogenous levels of active MMP-1 in samples, do not add APMA to the sample wells. Add 200 µL Reagent Diluent 2 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 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 protected completely from light 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 and emission wavelength set to 405 nm; endpoint mode; 1 x 20 mS integration time; plate speed = 6.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, working standards, and samples as instructed.

2. Add 100 µL of Assay Diluent RD1-64 to each well.

3. Add 150 µL of Standard, sample, or control to each well. Incubate 3 hours at RT on the shaker.

4. Aspirate and wash 4 times.

5. Add 200 µL of APMA to all standard and desired sample wells.* Incubate 2 hours at 37° C in a humidified environment.

6. Aspirate and wash 4 times.

7. Add 200 µL of Substrate to each well. Incubate 17 - 20 hours at 37° C in a humidified environment. **Protect from light within 10 minutes of Substrate addition.**

8. Determine RFU.
excitation λ 320 nm
emission λ 405 nm

*The addition of APMA will activate any potentially active forms of MMP-1 present in the sample. To measure endogenous levels of active MMP-1 in samples, do not add APMA to the sample wells. Add 200 µL Reagent Diluent 2 to these sample wells instead. APMA must always be added to the standard wells.

CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard RFU.

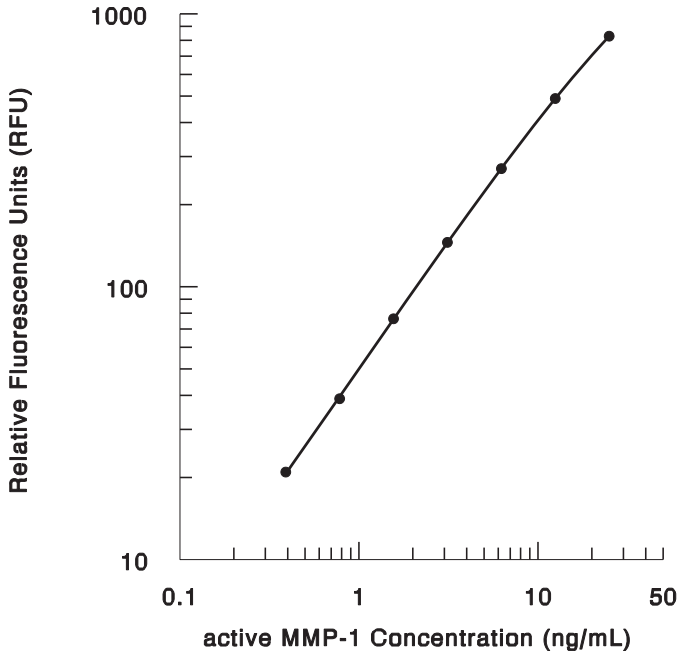
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve fit. As an alternative, construct a standard curve by plotting the mean RFU for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the active MMP-1 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.

To determine the active MMP-1 concentration of each sample, first find the RFU value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding active MMP-1 concentration.

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

TYPICAL DATA

This standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.



ng/mL	RFU	Average	Corrected
0	19.69 19.60	19.65	—
0.39	40.51 40.66	40.59	20.94
0.78	58.73 58.34	58.53	38.89
1.56	93.78 98.23	96.0	76.36
3.12	164.0 166.1	165.0	145.4
6.25	292.3 289.0	290.7	271.0
12.5	510.5 507.7	509.1	489.5
25	849.6 847.0	848.3	828.7

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 toweling in a sealed container.

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 seventy-eight separate assays to assess inter-assay precision.

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	78	78	78
Mean (ng/mL)	2.01	5.22	15.7	2.07	5.99	14.8
Standard deviation	0.21	0.52	1.50	0.37	0.52	1.32
CV (%)	10.4	10.0	9.6	17.7	8.7	8.9

RECOVERY

Samples were spiked with pro-MMP-1 to three different levels throughout the range of the assay. Samples were activated during the assay with the addition of APMA and the recovery of active MMP-1 was evaluated.

Sample Type	Average % Recovery	Range
Cell Culture Supernate (n=4)	97	89 - 107%
Serum (n=5)	100	93 - 104%
Heparin Plasma (n=5)	101	92 - 110%

LINEARITY

To assess the linearity of the assay, samples were spiked with high concentrations of pro-MMP-1 and diluted with Calibrator Diluent RD5-25 to produce sample values within the dynamic range of the assay. Samples were activated during the assay with the addition of APMA.

		Cell Culture Supernate (n=5)	Serum (n=5)	Heparin plasma (n=5)
1:2	Average % of Expected	99	103	100
	Range (%)	93-105	98-108	97-102
1:4	Average % of Expected	96	99	101
	Range (%)	81-107	93-111	95-108
1: 8	Average % of Expected	98	98	96
	Range (%)	93-106	93-108	91-105
1:16	Average % of Expected	104	94	93
	Range (%)	102-111	80-107	85-104

SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of active MMP-1 ranged from 0.01 ng/mL to 0.15 ng/mL. The mean MDD was 0.052 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.

CALIBRATION

This assay is calibrated against a highly purified NS0-expressed recombinant human MMP-1 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of both endogenous active MMP-1 (no APMA) and all potentially active forms of MMP-1 (APMA) in this assay. No medical histories were available for the donors used in this study. All samples tested for endogenous levels of active MMP-1 measured less than the lowest MMP-1 standard, 0.39 ng/mL.

	MMP-1 (APMA activated)		
Sample Type	Range (ng/mL)	% Detectable	Mean of Detectable (ng/mL)
Serum (n=36)	0.88 - 12.1	100	6.49
Heparin Plasma (n=36)	ND - 2.47	83	1.34

ND = Non-detectable, < 0.39 ng/mL

Cell Culture Supernates - Human peripheral blood lymphocyte cells (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 supernates were removed and assayed for levels of active MMP-1 (APMA activated).

	MMP-1 (APMA activated)	
Condition	Day 1 (ng/mL)	Day 5 (ng/mL)
Unstimulated	0.55	0.94
Stimulated	1.66	7.03

HT-1080 human fibrosarcoma cells were cultured in DMEM supplemented with 10% fetal calf serum. The cells were cultured for 4 days unstimulated or stimulated with 50 ng/mL PMA and 500 ng/mL calcium ionomycin. Aliquots of the supernates were removed and assayed for levels of active MMP-1 (APMA activated) and endogenous active MMP-1 (no APMA).

	MMP-1 (APMA activated)	endogenous active MMP-1 (no APMA)
Unstimulated	1.86 ng/mL	ND
Stimulated	478 ng/mL	0.934 ng/mL

ND = Non-detectable

SPECIFICITY

This assays recognizes both natural and recombinant human active MMP-1.

Cross-reactivity:

The factors listed below were prepared at 250 ng/mL in Calibrator Diluent RD5-25 and assayed for cross-reactivity. No significant cross-reactivity was observed.

Reconbinant human:
MMP-2
MMP-3
MMP-7
MMP-8
MMP-9
MMP-10
MMP-12
MMP-13
TIMP-1
TIMP-2

Interference:

Recombinant human TIMP-1 and TIMP-2 were spiked into a mid-range MMP-1 control and assayed. Interference was observed at concentrations ≥ 62.5 ng/mL.

TIMP-1 Interference			**TIMP-2 Interference**	
TIMP-1 Concentration (ng/mL)	Observed MMP-1 Value (ng/mL)		TIMP-2 Concentration (ng/mL)	Observed MMP-1 value (ng/mL)
250	3.29		250	2.85
125	5.70		125	3.33
62.5	6.01		62.5	5.48
0	5.89		0	5.89

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PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.