Quantikine® ELISA

Human MMP-9 Immunoassay

Catalog Number DMP900
SMP900
PDMP900

For the quantitative determination of human active (82 kDa) and Pro- (92 kDa) Matrix Metalloproteinase 9 (MMP-9) concentrations in cell culture supernates, serum, 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

Matrix metalloproteinases (MMPs), also called matrixins, constitute a family of zinc and calcium dependent endopeptidases that function in the breakdown of the extracellular matrix (ECM) and in the processing of a variety of molecules in different subcellular environments. They play an important role in many normal physiological processes such as embryonic development, morphogenesis, reproduction, and tissue remodeling (1, 2). They also participate in inflammatory and autoimmune disorders such as arthritis, cancer, and cardiovascular disease (3-5). 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, α2-Macroglobulin, and tissue inhibitors of metalloproteinases (TIMPs) (6).

MMP-9 (also referred to as gelatinase B, 92 kDa type IV collagenase, 92 kDa gelatinase, and type V collagenase) is secreted as a glycosylated proenzyme (6-8). Activation of the proenzyme involves proteolytic removal of the N-terminal pro region, resulting in the 82 kDa active enzyme (9, 10). Active human MMP-9 shares 72% and 74% amino acid sequence identity with mouse and rat MMP-9, respectively. In addition to the zinc-binding site, the catalytic domain also contains three contiguous fibronectin type II homology units responsible for binding gelatin (11). A proline-rich hinge region links the catalytic domain to the C-terminal hemopexin-like domain. *In vitro* treatment of the proenzyme with 4-aminophenylmercuric acetate (APMA) produces not only the active enzyme but also a C-terminal truncated form with activity comparable to that of the active form (12). MMP-9 degrades components of the ECM with high specific activity for denatured collagens (gelatin). It can cleave native collagens of type III, IV, V, and XI, as well as Elastin, Nidogen-1, and Vitronectin (2, 3). MMP-9 can also cleave a variety of chemokines and growth factors (e.g. IL-1β, CXCL8/IL-8, CXCL7, CXCL4, CXCL1, Latent TGF-β, membrane bound TNF-α, VEGF, and FGF basic), Amyloid β peptide, Substance P, and Myelin Basic Protein (3, 13-15). This action can increase or decrease the biological activity of soluble factors and can also liberate them from association with the ECM (16, 17). MMP-9 can also trigger signaling through various transmembrane proteins or inhibit signaling by inducing their shedding from the cell surface (e.g. CD44, E-Cadherin, Integrins, ICAM-1, and IL-2 Rα) (3, 18-20).

MMP-9 is produced by a variety of normal and transformed cells including neutrophils, monocytes, macrophages, astrocytes, fibroblasts, osteoclasts, chondrocytes, keratinocytes, endothelial and epithelial cells. It exerts physiological and pathological angiogenic and remodeling effects on the vasculature (21-25). Activated neutrophils release proMMP-9 which is free of TIMP-1, allowing the liberation of pro-angiogenic FGF-2 from the ECM (17). MMP-9 in complex with TIMP-1 does not induce FGF-2 release (17). Neutrophil-derived MMP-9 exacerbates the inflammatory response, in part by generating collagen-derived peptides that induce the release of additional neutrophil MMP-9 (26). MMP-9 also plays a role in bone formation and remodeling (1, 21, 27), methamphetamine-induced behavioral sensitization and reward (28), the regulation of neuronal synapse remodeling (29), trophoblast invasion during implantation (30), and the inactivation of Serpin α1-Proteinase Inhibitor (31). The shedding of adhesion proteins by MMP-9 has a direct effect on tumor cell invasiveness (18-20).

Circulating levels of MMP-9 are increased in many inflammatory disorders including intraluminal thrombus formation (32), atherosclerosis (33), Crohn's disease (34), hepatitis C virus infection (35), colorectal cancer (36), and Duchenne muscular dystrophy (37). The ratio of MMP-9 to TIMP-1 is also increased in multiple sclerosis serum (38) and cystic fibrosis sputum (39), but it is decreased in the serum during cytomegalovirus infection (40). Levels of free MMP-9 and complexes of MMP-9 with Lipocalin-2/NGAL are elevated in the urine of ovarian cancer and uterine tract infection patients, respectively (41, 42).

The Quantikine® Human MMP-9 Immunoassay is a 3.5 hour solid phase ELISA designed to measure MMP-9 (92 kDa Pro- and 82 kDa active forms, but not the 65 kDa form) in cell culture supernates, saliva, serum, plasma, and urine. It is calibrated with CHO-cell expressed recombinant human Pro-MMP-9, and the antibodies were raised against the recombinant factor. Natural human MMP-9 showed dose-response curves that were parallel to the standard curves obtained using the recombinant Quantikine® kit standards, indicating that this kit can be used to determine relative mass values of human MMP-9.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human MMP-9 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells, and MMP-9 is bound by the immobilized antibody. After washing away unbound substances, an enzyme-linked polyclonal antibody specific for human MMP-9 is added to the wells. Following a wash to remove unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of MMP-9 bound in the initial step. The color development is stopped and the intensity of the color is measured.

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. If cell culture supernate samples require large dilutions, perform an intermediate dilution with culture media and the final dilution with calibrator diluent.
- 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 Quantikine® Immunoassay, the possibility of interference cannot be excluded.

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.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

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

PART	PART #	CATALOG # DMP900	CATALOG # SMP900	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human MMP-9 Microplate	890613	1 plate	6 plates	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human MMP-9.	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.*
Human MMP-9 Standard	890615	2 vials	12 vials	Recombinant human Pro-MMP-9 in a buffered protein base with preservatives; lyophilized. *Refer to vial label for reconstitution volume.*	Discard the MMP-9 stock solution and dilutions after use. Use a fresh standard for each assay.
Human MMP-9 Conjugate	890614	1 vial	6 vials	21 mL/vial of a polyclonal antibody specific for human MMP-9 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1-34	895265	1 vial	6 vials	11 mL/vial of a buffered protein base with preservatives.	
Calibrator Diluent RD5-10	895266	3 vials	18 vials	21 mL/vial of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	1 vial	6 vials	21 mL/vial of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	1 vial	6 vials	12 mL/vial of stabilized hydrogen peroxide.	
Color Reagent B	895001	1 vial	6 vials	12 mL/vial of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	1 vial	6 vials	6 mL/vial of 2 N sulfuric acid.	
Plate Sealers	N/A	4 strips	24 strips	Adhesive strips.	

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

DMP900 contains sufficient materials to run an ELISA on one 96 well plate.
SMP900 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems®, Catalog # PDMP900). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Refer to the literature accompanying your order for specific vial counts.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Collection device for saliva samples which has no enzyme binding or filtering capabilities such as Salivette® or equivalent.
- 500 mL graduated cylinder.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human MMP-9 Controls (optional; R&D Systems®, Catalog # QC130).

PRECAUTIONS

MMP-9 is 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.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

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

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.

Platelet-Poor Plasma - Collect plasma on ice using heparin as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. An additional centrifugation step of the plasma at 10,000 x g for 10 minutes at 2-8 °C is recommended for complete platelet removal. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *EDTA and citrate are not recommended anticoagulants for use in this assay due to their chelating properties.*

MMP-9 is released upon platelet activation. To measure circulating levels of MMP-9, platelet-free plasma should be used for measurement. It should be noted that many protocols for plasma preparation, including procedures recommended by the Clinical Laboratory and 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 using a collection device such as a Salivette or equivalent. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Saliva collector cannot have any enzyme binding or filtering capabilities.*

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.

Serum, cell culture supernate, and saliva samples require at least a 100-fold dilution into Calibrator Diluent RD5-10. A suggested 100-fold dilution is 10 μL of sample + 990 μL of Calibrator Diluent RD5-10.

Platelet-poor plasma samples require at least a 40-fold dilution into Calibrator Diluent RD5-10. A suggested 40-fold dilution is 10 μL of sample + 390 μL of Calibrator Diluent RD5-10.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

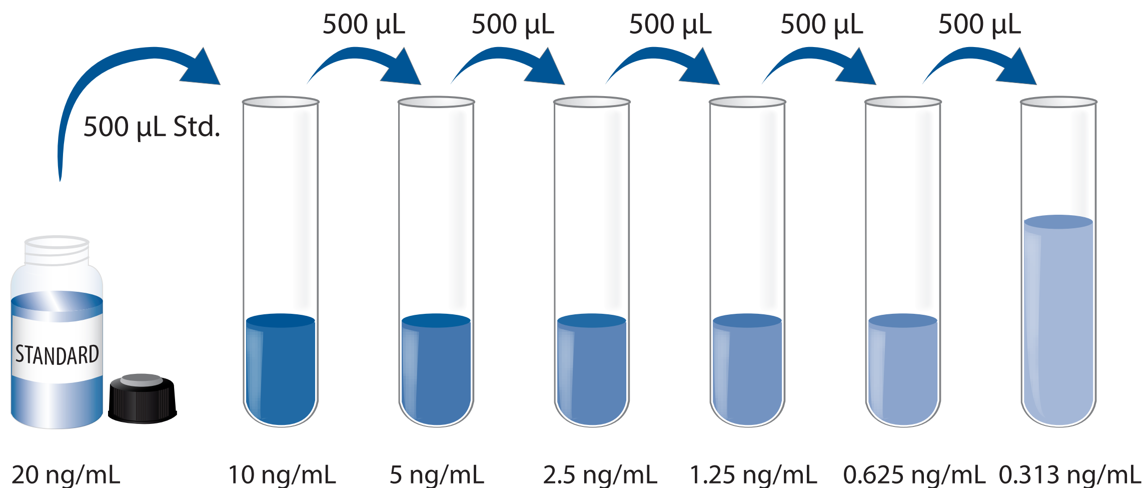
Note: *High concentrations of MMP-9 are found in saliva. Use a face mask and gloves to protect kit reagents from contamination.*

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.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Human MMP-9 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human MMP-9 Standard with deionized or distilled water. This reconstitution produces a stock solution of 20 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 RD5-10 into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human MMP-9 Standard (20 ng/mL) serves as the high standard. Calibrator Diluent RD5-10 serves as the zero standard (0 ng/mL).



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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.

Note: *High concentrations of MMP-9 are found in saliva. Use a face mask and gloves to protect the assay from contamination.*

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 RD1-34 to each well.
4. Add 100 µL of standard, control, 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. 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 towels.
6. Add 200 µL of Human MMP-9 Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature on the shaker.
7. Repeat the aspiration/wash as in step 5.
8. Add 200 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the benchtop. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

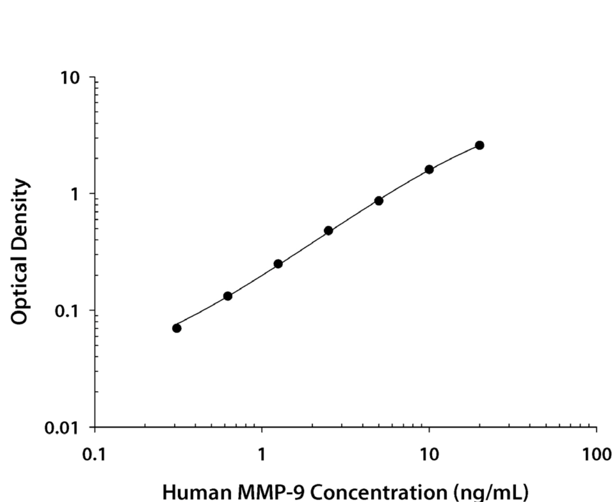
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 human MMP-9 concentrations versus the log of the O.D. 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

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



(ng/mL)	O.D.	Average	Corrected
0	0.040 0.041	0.040	—
0.313	0.111 0.110	0.110	0.070
0.625	0.172 0.171	0.172	0.132
1.25	0.292 0.288	0.290	0.250
2.5	0.532 0.508	0.520	0.480
5	0.910 0.899	0.904	0.864
10	1.612 1.668	1.640	1.600
20	2.607 2.638	2.622	2.582

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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 forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (ng/mL)	0.833	2.04	11.0	0.972	2.35	12.2
Standard deviation	0.017	0.039	0.316	0.077	0.184	0.845
CV (%)	2.0	1.9	2.9	7.9	7.8	6.9

RECOVERY

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

Sample Type	Average % Recovery	Range (ng/mL)
Cell culture media* (n=5)	97	85-104%
Serum* (n=5)	95	91-99%
Platelet-poor heparin plasma* (n=5)	96	89-108%
Saliva* (n=4)	90	74-114%
Urine (n= 5)	91	82-99%

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

SENSITIVITY

The minimum detectable dose (MDD) of human MMP-9 is typically less than 0.156 ng/mL.

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

LINEARITY

To assess the linearity of the assay, samples spiked with or containing high concentrations of human MMP-9 were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media* (n=5)	Serum* (n=5)	Platelet-poor Heparin plasma* (n=5)	Saliva* (n=4)	Urine (n=4)
1:2	Average % of Expected	104	103	102	105	101
	Range (%)	101-107	99-107	98-107	103-106	96-109
1:4	Average % of Expected	104	103	102	108	103
	Range (%)	101-106	99-108	99-106	103-109	95-110
1:8	Average % of Expected	102	102	104	111	104
	Range (%)	97-110	96-108	101-110	108-114	94-112
1:16	Average % of Expected	102	102	103	110	97
	Range (%)	96-110	95-108	95-110	109-112	90-109

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

CALIBRATION

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

SAMPLE VALUES

Serum/Platelet-Poor Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human MMP-9 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean of Detectable (ng/mL)	% Detectable	Range (ng/mL)
Serum (n=37)	436	100	169-705
Platelet-poor heparin plasma (n=37)	32	100	13.2-105
Saliva (n=4)	201	100	102-543
Urine (n=53)	3.65	45	ND-33.6

ND=Non-detectable

Cell Culture Supernates - Human peripheral blood mononuclear cells (5 x 10^6 cells/mL) were cultured in RPMI supplemented with 5% fetal bovine 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 on days 1 and 5 and assayed for levels of human MMP-9.

Condition	Day 1 (ng/mL)	Day 5 (ng/mL)
Unstimulated	132	33.8
Stimulated	522	210

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SPECIFICITY

This assay measures natural and recombinant 92 kDa Pro-MMP-9 and the 82 kDa active MMP-9. It does not measure the 65 kDa form.

The factors listed below were prepared at 100 or 200 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 200 ng/mL in a mid-range recombinant human MMP-9 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

ADAM8
ADAM10
ADAM15
Lipocalin-2/NGAL
MMP-1
MMP-2
MMP-3
MMP-7
MMP-8
MMP-10
MMP-12 (catalytic domain)
MMP-13
TACE (ADAM17)
TIMP-2
TIMP-3
TIMP-4

Recombinant mouse:

ADAM9
ADAM10
ADAM15
Lipocalin-2/NGAL
MMP-2
MMP-3
MMP-9
TIMP-1

No cross-reactivity was observed with recombinant human TIMP-1, but interference was observed at concentrations ≥ 6.25 ng/mL.

rhTIMP-1 Concentration (ng/mL)	Observed Value (ng/mL)
200	1.241
100	2.026
50	2.920
25	3.651
12.5	4.655
6.25	5.219
0	5.633

Interference was also observed with recombinant rat TIMP-1 at concentrations ≥ 200 ng/mL.

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PLATE LAYOUT

Use this plate layout to record standards and samples assayed.

12
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1
A B C D E F G H

NOTES

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