ELISA Kit
Catalog # KHC3061

Human
MMP-9

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PURPOSE

The Invitrogen Human MMP-9 (Hu MMP-9) ELISA is to be used for the quantitative determination of Hu MMP-9 in serum, plasma, buffered solution and tissue culture medium. The assay will recognize both natural and recombinant Hu MMP-9.

INTRODUCTION

Matrix metalloproteinase (MMP) is a class of zinc-dependent proteolytic enzymes that are responsible for the degradation of extracellular matrix (ECM) proteins. To date, more than 20 different human MMPs have been cloned and characterized. According to their structure, substrate specificity, and cellular localization, they can be classified into several subgroups, gelatinases (MMP-2 and MMP-9), interstitial collagenases (MMP-1, MMP-8, MMP-13 and MMP-18), stromelysins (MMP-3 and MMP-10), matrilysin (MMP-7), metalloelastases (MMP-12), and membrane type MMPs (MT-MMPs). The expression of MMPs is transcriptionally regulated by growth factors, hormones, cytokines, and cellular transformation. Most MMPs, except MT-MMPs, are synthesized as pre-proenzymes and secreted as inactive zymogens into the extracellular matrix, where subsequent activation results in cleavage of the proenzymes into the active species. The proteolytic activities of MMPs are precisely controlled during activation from their precursors and inhibition by endogenous inhibitors, α-macroglobulins and tissue inhibitors of metalloproteinases (TIMPs).

MMP-9, also known as gelatinase B, 92 kDa gelatinase, and 92 kDa type IV collagenase, represents the largest and most complex member of MMP family. MMP-9 comprises five distinct domains: a

propeptide domain which is cleaved upon activation, a gelatin-binding domain consisting of three contiguous fibronectin type II units, a catalytic domain containing the zinc binding motif, a proline-rich linker region, and a carboxyl terminal hemopexin-like domain. Like other MMPs, MMP-9 is first synthesized as inactive proenzyme. Activation of proMMP-9 is mediated by plasminogen activator/plasmin system and other MMPs. The regulation of MMP-9 activity is also controlled through TIMPs.

MMP-9 is produced by many types of cells, such as monocytes, macrophages, neutrophils, keratinocytes, fibroblasts, osteoclasts, chondrocytes, endothelial cells, and various tumor cells. Expression of MMP-9 is regulated by several cytokines and growth factors, including interleukins, interferons, EGF, NGF, FGF, VEGF, PDGF, TNF-α, TGF-β, the extracellular matrix metalloproteinase inducer EMMPRIN and also osteopontin. Upon activation, MMP-9 acts on a broad range of substrates including gelatin, collagen types IV, V, XI, and XVI, elastin, decorin, fibrillin, laminin and also activates growth factors like proTGF-β and proTNF-α. Physiologically, MMP-9 in coordination with other MMPs, play a role in normal tissue remodeling events such as neurite gowth, embryonic development, angiogenesis, ovulation, mammary gland involution and wound healing. MMP-9 with other MMPs is also involved in osteoblastic bone formation and/or inhibits osteoclastic bone resorption.

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Read entire protocol before use.

PRINCIPLE OF THE METHOD

The Invitrogen Hu MMP-9 kit is a solid phase sandwich Enzyme Linked-Immuno-Sorbent Assay (ELISA). A highly purified antibody has been coated onto the wells of the microtiter strips provided.

During the first incubation, standards of known Hu MMP-9 content, controls, and unknown samples are pipetted into the coated wells. After washing, biotinylated second antibody, is added.

After washing, Streptavidin-Peroxidase (enzyme) is added. This binds to the biotinylated antibody to complete the four-member sandwich. After a third incubation and washing to remove all the unbound enzyme, a substrate solution is added, which is acted upon by the bound enzyme to produce color. The intensity of this colored product is directly proportional to the concentration of Hu MMP-9 present in the original specimen.

REAGENTS PROVIDED

Note: *Store all reagents at 2 to 8°C.*

Reagent	*96 Test Kit*
Hu MMP-9 Standard, purified Hu MMP-9. Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume.	2 vials
Standard Diluent Buffer. Contains 0.1% sodium azide; 25 mL per bottle.	1 bottle
Hu MMP-9 Antibody Coated Wells. 96 Well Plate.	1 plate
Hu MMP-9 Biotin Conjugate, (Biotin-labeled anti-Hu MMP-9). Contains 0.1% sodium azide; 11 mL per bottle.	1 bottle
Streptavidin-HRP (100X). Contains 3.3 mM thymol; 0.125 mL per vial.	1 vial
Streptavidin-HRP Diluent. Contains 3.3 mM thymol; 25 mL per bottle.	1 bottle
Wash Buffer Concentrate (25X); 100 mL per bottle.	1 bottle
Stabilized Chromogen, *Tetramethylbenzidine (TMB);* 25 mL per bottle.	1 bottle
Stop Solution; 25 mL per bottle.	1 bottle
Plate Covers, adhesive strips.	4

Disposal Note: This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state and local regulations for disposal.

SUPPLIES REQUIRED BUT NOT PROVIDED

1. Microtiter plate reader capable of measurement at or near 450 nm.
2. Calibrated adjustable precision pipettes, preferably with disposable plastic tips. (A manifold multi-channel pipette is desirable for large assays.)
3. Distilled or deionized water.
4. Plate washer: automated or manual (squirt bottle, manifold dispenser, etc.).
5. Data analysis and graphing software. Graph paper: linear (Cartesian), log-log, or semi-log, as desired.
6. Glass or plastic tubes for diluting and aliquoting standard.
7. Absorbent paper towels.
8. Calibrated beakers and graduated cylinders in various sizes.

PROCEDURAL NOTES/LAB QUALITY CONTROL

1. When not in use, kit components should be refrigerated. All reagents should be warmed to room temperature before use.
2. **Microtiter plates should be allowed to come to room temperature before opening the foil bags.** Once the desired number of strips has been removed, immediately reseal the bag and store at 2 to 8°C to maintain plate integrity.
3. Samples should be collected in pyrogen/endotoxin-free tubes.
4. Samples should be frozen if not analyzed shortly after collection. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well prior to analysis.
5. When possible, avoid use of badly hemolyzed or lipemic serum and plasma samples. If large amounts of particulate matter are present, centrifuge or filter prior to analysis.

6. It is recommended that all standards, controls and samples be run in duplicate.
7. Serum, plasma, or tissue culture sample(s) that measure >1,500 pg/mL require additional dilution steps in their respective buffers.
8. When pipetting reagents, maintain a consistent order of addition from well-to-well. This ensures equal incubation times for all wells.
9. Cover or cap all reagents when not in use.
10. **Do not mix or interchange different reagent lots from various kit lots**.
11. Do not use reagents after the kit expiration date.
12. Read absorbances within 30 minutes of assay completion.
13. In-house controls provided should be run with every assay. If control values fall outside pre-established ranges, the accuracy of the assay is suspect.
14. All residual wash liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. ***Never*** insert absorbent paper directly into the wells.
15. Because *Stabilized Chromogen* is light sensitive, avoid prolonged exposure to light. Also avoid contact between *Stabilized Chromogen* and metal, or color may develop.

SAFETY

All blood components and biological materials should be handled as potentially hazardous. Follow universal precautions as established by the Centers for Disease Control and Prevention and by the Occupational Safety and Health Administration when handling and disposing of infectious agents.

DIRECTIONS FOR WASHING

Incomplete washing will adversely affect the test outcome. All washing must be performed with the *Wash Buffer Concentrate (25X)* provided.

Washing can be performed manually as follows: completely aspirate the liquid from all wells by gently lowering an aspiration tip (aspiration device) into the bottom of each well. Take care not to scratch the inside of the well.

After aspiration, fill the wells with at least 0.4 ml of diluted wash solution. Let soak for 15 to 30 seconds, then aspirate the liquid. Repeat as directed under **ASSAY METHOD**. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

Alternatively, the wash solution may be put into a squirt bottle. If a squirt bottle is used, flood the plate with wash buffer, completely filling all wells. After the washing procedure, the plate is inverted and tapped dry on absorbent tissue.

If using an automated washer, the operating instructions for washing equipment should be carefully followed.

SAMPLE PREPARATION

Human serum and plasma require a 80-fold dilution in the *Standard Diluent Buffer*. For these samples, first prepare a 1:10 dilution by adding 10 µL of the sample to a clean microfuge tube, followed by 90 µL of *Standard Diluent Buffer*. Mix well. Next, prepare an additional 1:8 dilution by transferring 15 µL from the first tube into a second clean microfuge tube, followed by the addition of 105 µL of *Standard Diluent Buffer*. Mix well.

Tissue culture samples require a 40-fold dilution in the *Standard Diluent Buffer*. For these samples, add 117 µL of *Standard Diluent Buffer* to a clean microfuge tube, followed by 3 µL of the sample. Mix well.

REAGENT PREPARATION AND STORAGE

A. Reconstitution and Dilution of Hu MMP-9 Standard

The *Hu MMP-9 Standard* is prepared from a highly purified *human MMP-9* protein.

Note: Either glass or plastic tubes may be used for standard dilutions.

1. Reconstitute standard to 1,500 pg/mL with *Standard Diluent Buffer*. Refer to the standard vial label for instructions. Swirl or mix gently and allow to sit for 10 minutes to ensure complete reconstitution. Use the standard within 15 minutes of reconstitution.
2. Add 0.300 mL of *Standard Diluent Buffer* to each of 6 tubes labeled 750, 375, 187.5, 93.8, 46.9, and 23.5 pg/mL Hu MMP-9.
3. Make serial dilutions of the standard as described in the following dilution table. Mix thoroughly between steps.

B. Dilution of Hu MMP-9 Standard

Standard:	Add:	Into:
1,500 pg/mL	Prepare as described in Step 1.	
750 pg/mL	0.300 mL of the 1,500 pg/mL std.	0.300 mL of the Diluent Buffer
375 pg/mL	0.300 mL of the 750 pg/mL std.	0.300 mL of the Diluent Buffer
187.5 pg/mL	0.300 mL of the 375 pg/mL std.	0.300 mL of the Diluent Buffer
93.8 pg/mL	0.300 mL of the 187.5 pg/mL std.	0.300 mL of the Diluent Buffer
46.9 pg/mL	0.300 mL of the 93.8 pg/mL std.	0.300 mL of the Diluent Buffer
23.5 pg/mL	0.300 mL of the 46.9 pg/mL std.	0.300 mL of the Diluent Buffer
0 pg/mL	0.300 mL of the Diluent Buffer	An empty tube

Discard all remaining reconstituted and diluted standards after completing assay. Return the *Standard Diluent Buffer* to the refrigerator.

C. Storage and Final Dilution of Streptavidin-HRP (100X)

Please Note: The *Streptavidin-HRP (100X)* is in 50% glycerol. This solution is viscous. To ensure accurate dilution, allow *Streptavidin-HRP (100X)* to reach room temperature. Gently mix. Pipette *Streptavidin-HRP (100X)* slowly. Remove excess concentrate solution from pipette tip by gently wiping with clean absorbent paper.

1. Within 15 minutes of use, dilute 10 µL of this 100X concentrated solution with 1 mL of *Streptavidin-HRP Diluent* for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution.

For Example:

# of 8-Well Strips	Volume of Streptavidin-HRP (100X)	Volume of Diluent
2	20 µL solution	2 mL
4	40 µL solution	4 mL
6	60 µL solution	6 mL
8	80 µL solution	8 mL
10	100 µL solution	10 mL
12	120 µL solution	12 mL

2. Return the unused *Streptavidin-HRP (100X)* to the refrigerator.

D. Dilution of Wash Buffer

Allow the *Wash Buffer Concentrate (25X)* to reach room temperature and mix to ensure that any precipitated salts have redissolved. Dilute 1 volume of the *Wash Buffer Concentrate (25X)* with 24 volumes of deionized water (e.g., 50 mL may be diluted up to 1.25 liters, 100 mL may be diluted up to 2.5 liters). Label as Working Wash Buffer.

Store both the concentrate and the Working Wash Buffer in the refrigerator. The diluted buffer should be used within 14 days.

ASSAY METHOD: PROCEDURE AND CALCULATIONS

Be sure to read the *Procedural Notes/Lab Quality Control* section before carrying out the assay.

Allow all reagents to reach room temperature before use. Gently mix all liquid reagents prior to use.

Note: A standard curve must be run with each assay.

1. Determine the number of 8-well strips needed for the assay. Insert these in the frame(s) for current use. (Re-bag extra strips and frame. Store these in the refrigerator for future use.)
2. Dilute serum and plasma samples 1:80, Tissue culture samples 1:40 with *Standard Diluent Buffer*. (see **SAMPLE PREPARATION** section on page 11).
3. Add 100 µL of the *Standard Diluent Buffer* to the zero standard wells. Well(s) reserved for chromogen blank should be left empty.
4. Add 100 µL of standards, controls, or samples (serum, plasma and tissue culture samples prediluted) to the appropriate microtiter wells.
5. Cover plate with *plate cover* and incubate for **2 hours at room temperature**.

6. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
7. Add 100 µL of biotinylated *Hu MMP-9 Biotin Conjugate* solution into each well except the chromogen blank(s).
8. Cover plate with *plate cover* and incubate for **1 hour at room temperature**.
9. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
10. Add 100 µL of Streptavidin-HRP Working Solution to each well except the chromogen blank(s). (Prepare the working dilution as described in **REAGENT PREPARATION AND STORAGE**, Section C.)
11. Cover plate with the *plate cover* and incubate for **30 minutes at room temperature**.
12. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. See **DIRECTIONS FOR WASHING**.
13. Add 100 µL of *Stabilized Chromogen* to each well. The liquid in the wells will begin to turn blue.
14. Incubate for **30 minutes at room temperature and in the dark**. ***Please Note*: Do not cover the plate with aluminum foil or metalized mylar**. The incubation time for chromogen substrate is often determined by the microtiter plate reader used. Many plate readers have the capacity to record a maximum optical density (O.D.) of 2.0. The O.D. values should be monitored and the substrate reaction stopped before the O.D. of the positive wells exceed the limits of the instrument. The O.D. values at 450 nm can only be read after the *Stop Solution* has been added to each well. If using a reader that records only to 2.0 O.D., stopping the assay after 20 to 25 minutes is suggested.

15. Add 100 µL of *Stop Solution* to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow.
16. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of *Stabilized Chromogen* and *Stop Solution*. Read the plate within 30 minutes after adding the *Stop Solution*.
17. Plot on graph paper the absorbance of the standards against the standard concentration. (Optimally, the background absorbance may be subtracted from *all* data points, including standards, unknowns and controls, prior to plotting.) Draw the best smooth curve through these points to construct the standard curve. If using curve fitting software, the four parameter algorithm provides the best curve fit.
18. Read the Hu MMP-9 concentrations for unknown samples and controls from the standard curve plotted in step 17. **Multiply value(s) obtained for serum, plasma and tissue culture samples by 80 or 40 to correct for the overall 1:80 or 1:40 dilution in step 2.** Samples producing signals greater than that of the highest standard (1,500 pg/mL) should be further diluted in the *Standard Diluent Buffer* as described in step 2 and reanalyzed. Multiply the concentration found by the appropriate dilution factor.

TYPICAL DATA

The following data were obtained for the various standards over the range of 0 to 1,500 pg/mL Hu MMP-9.

Standard Hu MMP-9 (pg/mL)	Optical Density (450 nm)
0	0.149
	0.143
23.5	0.267
	0.258
46.9	0.369
	0.368
93.8	0.560
	0.569
187.5	0.927
	0.943
375	1.571
	1.594
750	2.374
	2.483
1,500	3.179
	3.197

LIMITATIONS OF THE PROCEDURE

Do not extrapolate the standard curve beyond the 1,500 pg/mL standard point; the dose-response is non-linear in this region and accuracy is difficult to obtain. Dilute samples with concentrations exceeding the linear portion of the Standard Curve with the appropriate diluent buffer; reanalyze these and multiply results by the appropriate dilution factor.

The influence of various drugs, aberrant sera (hemolyzed, hyperlipidemic, jaundiced, etc.) and the use of biological fluids in place of serum samples have not been thoroughly investigated. The rate of degradation of native Hu MMP-9 in various matrices has not been investigated. The immunoassay literature contains frequent references to aberrant signals seen with some sera, attributed to heterophilic antibodies. Though such samples have not been seen to date, the possibility of this occurrence cannot be excluded.

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PERFORMANCE CHARACTERISTICS

SENSITIVITY

The minimum detectable dose of Hu MMP-9 is <10 pg/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times, and calculating the corresponding concentration.

PRECISION

1. Intra-Assay Precision

Samples of known MMP-9 concentration were assayed in replicates of 14 to determine precision within an assay.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	99.16	512.44	1004.62
SD	4.14	23.53	33.45
%CV	4.18	4.59	3.33

SD = Standard Deviation
CV = Coefficient of Variation

2. Inter-Assay Precision

Samples were assayed 42 times in multiple assays to determine precision between assays.

	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	99.28	544.34	1032.56
SD	7.94	42.24	71.02
%CV	8.00	7.76	6.88

SD = Standard Deviation
CV = Coefficient of Variation

LINEARITY OF DILUTION

Human serum, heparin plasma, and tissue culture medium spiked with Hu MMP-9 were serially diluted in *Standard Diluent Buffer* over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded average correlation coefficients of 0.999 for serum, 0.999 for heparin plasma, and 0.997 for tissue culture medium.

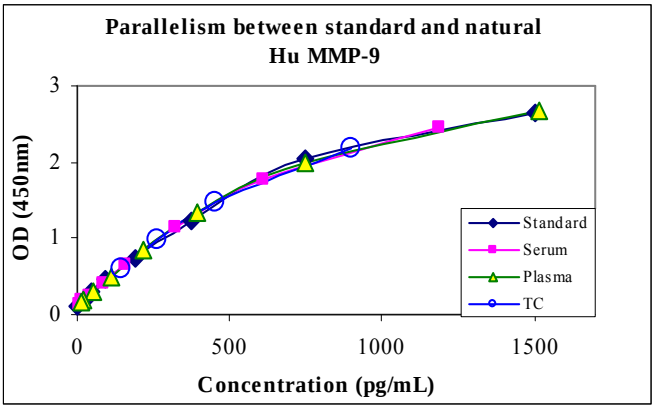
RECOVERY

The recoveries of purified Hu MMP-9 added to human serum, citrate plasma, heparin plasma, and tissue culture medium containing 10% fetal bovine serum were measured on the Invitrogen Hu MMP-9 ELISA.

Sample Type	Average % Recovery
Hu Serum	98
Hu Citrate plasma	88
Hu Heparin plasma	80
RPMI+10% fetal bovine serum	84
DMEM+10% fetal bovine serum	81
Serum and plasma samples were pre-diluted 80-fold, tissue culture medium samples were pre-diluted 40-fold, as described in sample preparation procedure.	

PARALLELISM

Random human serum, plasma and cell culture medium samples were serially diluted in the *Standard Diluent Buffer* and analyzed as described in **ASSAY METHOD**. The optical density of each dilution was plotted against the Hu MMP-9 standard curve. Parallelism was demonstrated by the figure below and indicated that the standard accurately reflects the Hu MMP-9 content in natural samples.



SPECIFICITY

Buffered solutions of a panel of substances ranging in concentrations from 1,320 to 132,000 pg/mL were assayed with the Invitrogen Hu MMP-9 kit and found to have no cross-reactivity: Human Eotaxin, GM-CSF, IFN-α, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 p40/p70, IL-13, IL-15, IL-17, IL-23, IP-10, MCP-1, MIG, MIP-1α, MIP-1β, MMP-2, MMP-13, RANTES, TIMP-1, TIMP-2, TIMP-4; Rat GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α; Mouse IL-1α, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IP-10, MIP-1α, KC, MCP-1, MMP-9, TNF-α, VEGF.

HIGH DOSE HOOK EFFECT

No hook effect was observed with concentrations up to 1 µg/mL.

EXPECTED VALUES

Thirty-two human serum and plasma samples, and un-stimulated and PMA stimulated HT1080 cell culture medium were evaluated for the presence of Hu MMP-9 in this assay.

Sample	Range (ng/mL)
Hu Serum (n=22)	22-71
Hu Citrate plasma (n=5)	22-38
Hu Heparin plasma (n=5)	24-37
HT1080 cell culture medium, un-stimulated	3-8
HT1080 cell culture medium, stimulated with 10nM, 80nM and 160nM PMA	21-94

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Explanation of symbols

Symbol	Description	Symbol	Description
REF	Catalogue Number	LOT	Batch code
RUO	Research Use Only	IVD	*In vitro* diagnostic medical device
	Use by		Temperature limitation
	Manufacturer	EC REP	European Community authorised representative
[-]	Without, does not contain	[+]	With, contains
	Protect from light		Consult accompanying documents
	Directs the user to consult instructions for use (IFU), accompanying the product.		

Human MMP-9 Assay Summary