Quantikine® ELISA

Human MMP-9/NGAL Complex Immunoassay

Catalog Number DM9L20

For the quantitative determination of human MMP-9/NGAL Complex concentrations in cell culture supernates, serum, plasma, urine, and saliva.

Note: The standard reconstitution method has changed. Please read this package insert in its entirety before using this product.

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 metalloproteinase 9 (MMP-9), also known as gelatinase B, 92 kDa type IV collagenase, 92 kDa gelatinase and type V collagenase, is a member of the MMP (matrixin) family that plays an important role in many normal physiological processes such as embryonic development, morphogenesis, reproduction and tissue remodeling (1, 2). MMPs also participate in many pathological processes such as arthritis, cancer and cardiovascular disease (3). As a zinc and calcium-dependent endopeptidase, MMP-9 cleaves a variety of substrates from the extracellular matrix such as gelatin, collagen types I, III, IV and V, elastin, aggrecan, galectin-3, and neonatal human proteoglycan link protein. In addition, it processes a number of non-matrix substrates such as myelin basic protein, substance P, amyloid β peptide, TGF-β2 and IL-1β precursors, IL-2 receptor type α, plasmin, specific variants of IL-8, connective tissue-activated peptide, platelet factor 4, and growth-related oncogene α (GROα/CXCL1). Secreted as a glycosylated pro-enzyme, the activity and localization of MMP-9 are regulated by several molecules including α2-macroglobulin, tissue inhibitors of metalloproteinases (TIMPs), CD44, α2(IV) chain of collagen IV and neutrophil gelatinase-associated lipocalin (NGAL) (1).

NGAL, also known as lipocalin-2, is a member of the lipocalin family that is comprised of functionally diverse but structurally conserved small proteins (4). It has been implicated in a variety of processes including inflammation, apoptosis, and organogenesis, and is a promising biomarker for acute renal failure (5-8). NGAL biosynthesis is stimulated by the Toll-like receptors on immune cells upon bacterial invasion; secreted NGAL then limits bacterial growth by sequestrating the iron-laden siderophore (9). NGAL exists as monomer (25 kDa), homodimer, and disulfide bond-linked heterodimer with MMP-9 (10). The MMP-9/NGAL complex (125 kDa) is one of several MMP forms with high molecular weights (> 100 kDa) detected in the urine of cancer patients (11). NGAL is proposed to modulate MMP-9 activity by protecting it from degradation (12).

The Quantikine® Human MMP-9/NGAL Complex Immunoassay is a 4.5 hour solid phase ELISA designed to measure the MMP-9/NGAL complex in cell culture supernates, serum, plasma, urine, and saliva. It contains natural human MMP-9/NGAL as the standard. The antibodies were raised against recombinant human MMP-9 and recombinant human NGAL. This Quantikine® kit will not detect recombinant human MMP-9 or NGAL in their free forms.

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 any complexed MMP-9/NGAL present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human NGAL is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of complexed MMP-9/NGAL 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 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 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 #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Human MMP-9/NGAL Microplate	892887	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 the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Human MMP-9/NGAL Conjugate	892888	21 mL of monoclonal antibody specific for human NGAL conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human MMP-9/NGAL Standard	893199	Human MMP-9/NGAL in a buffered protein solution with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-87	895879	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-10	895266	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895032	6 mL of 2 N sulfuric acid.	
Plate Sealers	N/A	4 adhesive strips.	

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

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.
- 500 mL graduated cylinder.
- Collection device for saliva samples that has no enzyme binding or filtering capabilities such as Salivette® or equivalent.
- **Polypropylene** test tubes for dilution of standards and samples.

PRECAUTIONS

The MMP-9/NGAL Standard contains MMP-9/NGAL derived from human blood. The source material was tested at the donor level using FDA licensed methods and found to be non-reactive for anti-HIV-1/2, anti-HCV, and Hepatitis B surface antigen. As no testing can offer complete assurance of freedom from infectious agents, the Standard should be handled as if capable of transmitting infection.

MMP-9/NGAL Complex is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

The Stop Solution provided with this kit is an acid solution.

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.

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 plasma are not recommended anticoagulants for use in this assay due to their chelating properties.*

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer, and assay immediately or aliquot and store samples at ≤-20 °C. Avoid repeated freeze-thaw cycles.

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 samples require a 20-fold dilution. A suggested 20-fold dilution is 10 µL of sample + 190 µL of Calibrator Diluent RD5-10.

Plasma/Saliva samples require a 5-fold dilution. A suggested 5-fold dilution is 40 µL of sample + 160 µL of Calibrator Diluent RD5-10.

REAGENT PREPARATION

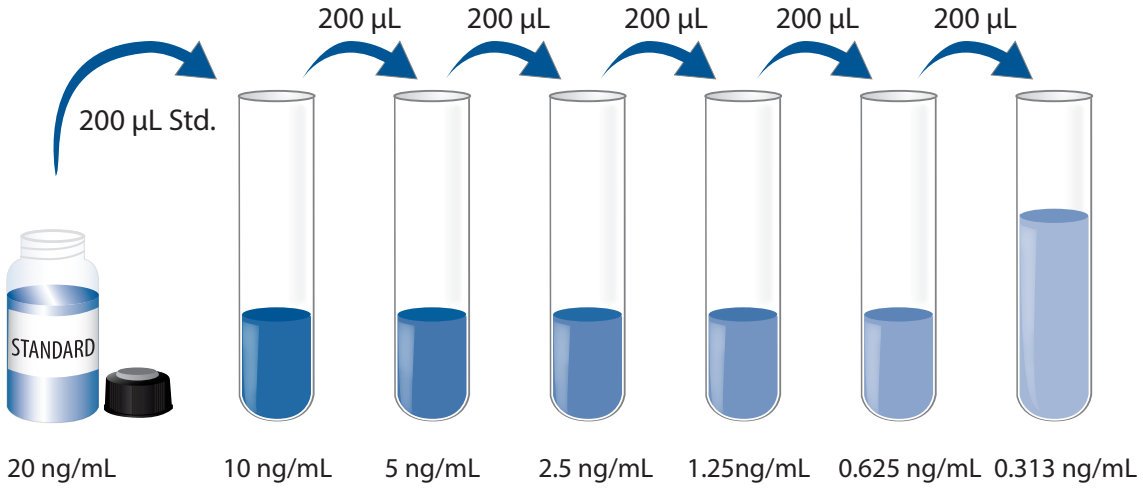
Bring all reagents to room temperature before use.

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/NGAL Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human MMP-9/NGAL Standard with deionized or distilled water. This reconstitution produces a stock solution of 20 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a 15-60 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 200 µL of Calibrator Diluent RD5-10 into each tube. Use the standard to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human MMP-9/NGAL Standard (20 ng/mL) serves as the high standard. Calibrator Diluent RD5-10 serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

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

Note: *High concentrations of MMP-9/NGAL Complex are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents 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 RD1-87 to each well.
4. Add 50 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 3 hours at room temperature.
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/NGAL Conjugate to each well. Cover with a new adhesive strip. Incubate for 1 hour at room temperature.
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

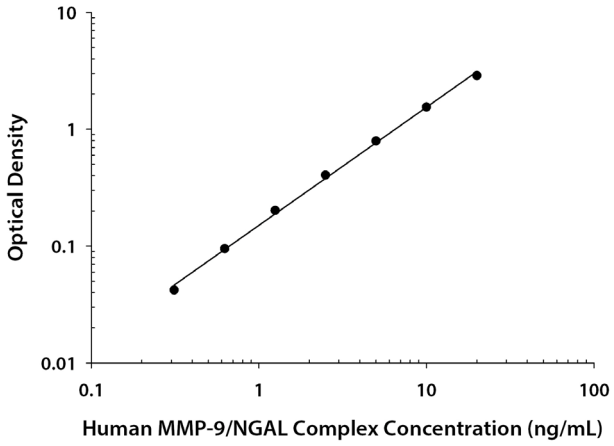
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a log/log 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 a log/log graph. The data may be linearized by plotting the log of the human MMP-9/NGAL concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If the 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	0.009 0.011	0.010	—
0.313	0.049 0.055	0.052	0.042
0.625	0.105 0.105	0.105	0.095
1.25	0.209 0.214	0.212	0.202
2.5	0.399 0.430	0.415	0.405
5	0.802 0.804	0.803	0.793
10	1.522 1.592	1.557	1.547
20	2.858 2.909	2.884	2.874

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	20	20	20
Mean (ng/mL)	1.84	5.52	10.3	1.70	5.13	9.92
Standard deviation	0.076	0.160	0.237	0.130	0.335	0.510
CV (%)	4.1	2.9	2.3	7.6	6.5	5.1

RECOVERY

The recovery of human MMP-9/NGAL spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	90	84-97%
Urine (n=4)	93	86-106%

LINEARITY

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

		Cell culture media (n=4)	Serum* (n=4)	Heparin plasma (n=4)	Saliva* (n=4)	Urine (n=5)
1:2	Average % of Expected	102	105	106	105	110
	Range (%)	93-108	102-110	99-111	97-112	106-117
1:4	Average % of Expected	103	109	99	105	110
	Range (%)	91-110	103-116	94-105	96-113	97-120
1:8	Average % of Expected	99	105	99	100	109
	Range (%)	89-112	96-111	97-104	91-115	97-125
1:16	Average % of Expected	97	105	97	104	105
	Range (%)	82-109	102-110	97-97	88-118	87-126

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

SENSITIVITY

Twenty assays were evaluated and the minimum detectable dose (MDD) of human MMP-9/NGAL ranged from 0.002-0.058 ng/mL. The mean MDD was 0.013 ng/mL.

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

CALIBRATION

This immunoassay is calibrated against a highly purified natural MMP-9/NGAL from human source material.

SAMPLE VALUES

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

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=35)	40.3	8.7-164	32.0
Heparin plasma (n=35)	6.35	1.92-44.8	8.00
Saliva (n=9)	51.4	7.38-109	33.0

Sample Type	Mean of Detectable (ng/mL)	% Detectable	Range (ng/mL)
Urine (n=14)	0.44	37.5	ND-0.67

ND=Non-detectable

Cell Culture Supernates - Two preparations of human neutrophils (1 x 10^7 cells/mL) were cultured in Hank's media and stimulated with 50 ng/mL of PMA for 30 minutes. An aliquot of each cell culture supernate was removed, assayed for levels of human MMP-9/NGAL Complex, and measured 480 ng/mL and 569 ng/mL, respectively.

SPECIFICITY

This assay recognizes natural and recombinant human MMP-9/NGAL Complex.

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

Recombinant human:

ADAM8
ADAM10
ADAM15
ADAMTS1
ADAMTSL1.2
Lipocalin-1
Lipocalin-2
MMP-1
MMP-2
MMP-3
MMP-7
MMP-8
MMP-9
MMP-10
MMP-12
MMP-13
MMP-14
MMP-16
TACE/ADAM17
TIMP-1
TIMP-2
TIMP-3
TIMP-4

Recombinant mouse:

ADAM9
ADAM10
ADAM15
ADAM33
Lipocalin-2
MMP-2
MMP-3
MMP-9
TIMP-1

Recombinant rat:

TIMP-1

Recombinant human MMP-9/TIMP-1 Complex interferes at concentrations > 25 ng/mL.

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