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R&D SYSTEMS

Quantikine® ELISA

Human TIMP-2 Immunoassay

Catalog Number DTM200

For the quantitative determination of human Tissue Inhibitor of Metalloproteinase 2 (TIMP-2) 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 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_2-macroglobulins and tissue inhibitors of metalloproteinases (TIMPs).

Among the four known members of the TIMP family, TIMP-2 has features both common to all the TIMPs and unique to itself (3). The common features include 12 cysteine residues that form 6 disulfide bonds, three of which are in the N- and C-terminal domain, respectively. The N-terminal domain is responsible for tight but non-covalent binding to the active MMPs in a 1:1 stoichiometry. The unique features of TIMP-2 include the binding of its C-terminal domain to the hemopexin-like domain of pro-MMP-2. This interaction is essential in the cell surface activation of pro-MMP-2 by active MMP-14 (MT1-MMP) (4, 5). TIMP-2 also has functions that are independent of MMP inhibition. For example, it suppresses EGF-mediated mitogenic signaling by short-circuiting EGF R activation (6).

TIMP-2 expression has been observed in both normal and tumor tissues (7). High levels of TIMP-2 correlate with both shortened disease-free interval and overall survival in human breast cancers (8). Serum levels of TIMP-2 are elevated in patients with systemic sclerosis, and correlate with the extent of skin sclerosis in these patients (9). TIMP-2 levels are reduced in ovarian carcinoma cells in effusions, in conjunction with increased MMP-2 levels (10). TIMP-2 levels are also reduced in tracheal aspirate fluid from preterm infants with respiratory distress, in conjunction with increased MMP-8 levels (11). TIMP-2 and TIMP-1 are down-regulated in stromal cells whereas pro-MMP-9 is enhanced in prostate cancer cells when the two types of cells are cultured together (12). TIMP-2 and TIMP-1 expression is low in grade II and III human brain tumors but significantly higher in grade I human brain tumors, indicating that their expression may be valuable markers for tumor malignancy (13). Recombinant human TIMP-2 delivered by an adenovirus vector inhibited tumor growth, angiogenesis and metastasis, and prolonged survival in mice (14).

The Quantikine® Human TIMP-2 Immunoassay is a 4.5 hour solid phase ELISA designed to measure human TIMP-2 levels in cell culture supernates, serum, plasma, saliva, and urine. It contains CHO cell-expressed recombinant human TIMP-2, and antibodies raised against the recombinant protein. Natural human TIMP-2 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 levels of human TIMP-2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human TIMP-2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TIMP-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human TIMP-2 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 TIMP-2 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, dilute the samples with the appropriate calibrator diluent and repeat the assay.
- Any variation in 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 enzymes 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 TIMP-2 Microplate	892158	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human TIMP-2.	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 TIMP-2 Conjugate	892159	21 mL of a polyclonal antibody specific for human TIMP-2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human TIMP-2 Standard	892160	Recombinant human TIMP-2 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1W	895117	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5P	895151	2 vials (21 mL/vial) of a buffered protein base with preservatives. *Use diluted 1:5 for cell culture supernates/saliva/urine. Use undiluted for serum/plasma.*	
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.
- 50 mL and 500 mL graduated cylinders.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- Collection device for saliva samples that has no enzyme binding or filtering capabilities such as Salivette® or equivalent.
- Test tubes for dilution of standards and samples.
- Human TIMP-2 Controls (optional; R&D Systems®, Catalog # QC174).

PRECAUTIONS

TIMP-2 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 SDS 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 EDTA or 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: *Citrate plasma has not been validated for use in this assay.*
Grossly hemolyzed samples are not suitable for use in this assay.

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

Serum and plasma samples require a 50-fold dilution. A suggested 50-fold dilution is 10 µL of sample + 490 µL of Calibrator Diluent RD5P.

Saliva samples require a 10-fold dilution. A suggested 10-fold dilution is 50 µL of sample + 450 µL of Calibrator Diluent RD5P (diluted 1:5).

Urine samples require a 2-fold dilution. A suggested 2-fold dilution is 150 µL of sample + 150 µL of Calibrator Diluent RD5P (diluted 1:5).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High levels of TIMP-2 are found in saliva. Take necessary precautions (e.g. mask and gloves) to protect kit reagents.*

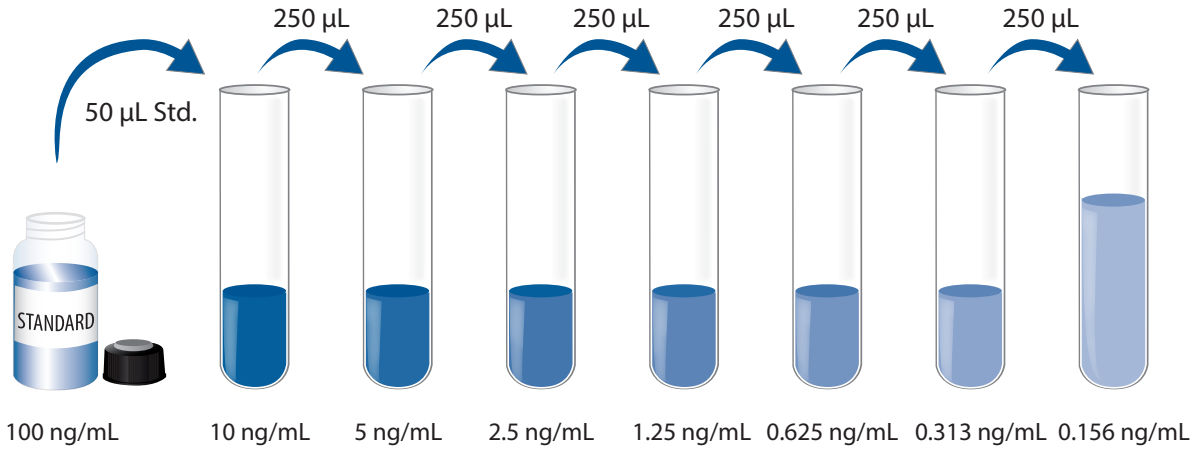
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to 480 mL of deionized or distilled water to prepare 500 mL of Wash Buffer.

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.

Calibrator Diluent RD5P (diluted 1:5) - **For cell culture supernate/saliva/urine samples only.** Add 10 mL of Calibrator Diluent RD5P to 40 mL of deionized or distilled water to prepare 50 mL of Calibrator Diluent RD5P (diluted 1:5).

Human TIMP-2 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human TIMP-2 Standard with deionized or distilled water. This reconstitution produces a stock solution of 100 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5P (diluted 1:5) (*for cell culture supernate/saliva/urine samples*) or Calibrator Diluent RD5P (*for serum/plasma samples*) into the 10 ng/mL tube. Pipette 250 µL of the appropriate calibrator diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 10 ng/mL standard serves as the high standard. The appropriate calibrator diluent 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 standards, controls, and samples be assayed in duplicate.

Note: *High levels of TIMP-2 are found in saliva. Take necessary precautions (e.g. mask and gloves) to protect kit reagents.*

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 RD1W to each well.
4. Add 50 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 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 TIMP-2 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours 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. **Protect from light.**
   For cell culture/saliva/urine samples: Incubate for 20 minutes at room temperature **on the benchtop.**
   For serum/plasma samples: Incubate for 30 minutes at room temperature **on the benchtop.**
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 if 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.).

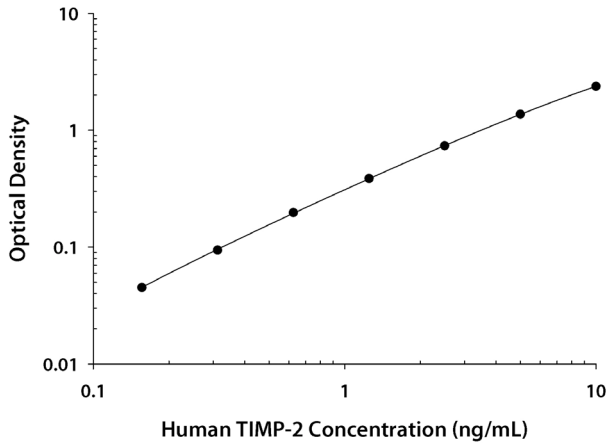
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw the best fit curve through the points on the graph. The data may be linearized by plotting the log of the human TIMP-2 concentrations versus the log of the optical density 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

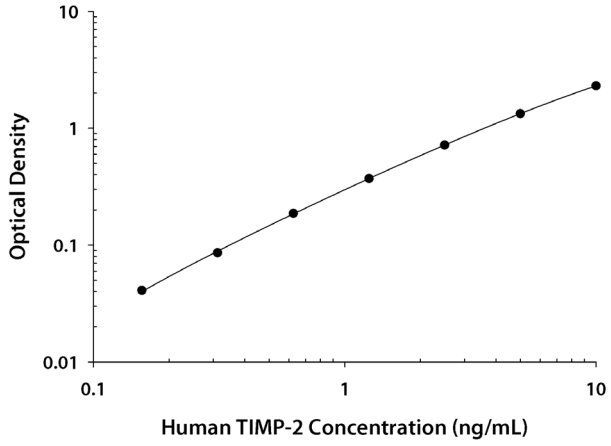
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE/SALIVA/URINE ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.013 0.013	0.013	—
0.156	0.057 0.059	0.058	0.045
0.313	0.105 0.109	0.107	0.094
0.625	0.205 0.214	0.210	0.197
1.25	0.387 0.410	0.399	0.386
2.5	0.739 0.759	0.749	0.736
5	1.358 1.403	1.381	1.368
10	2.354 2.423	2.389	2.376

SERUM/PLASMA ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.012 0.013	0.013	—
0.156	0.054 0.053	0.054	0.041
0.313	0.097 0.100	0.099	0.086
0.625	0.198 0.201	0.200	0.187
1.25	0.382 0.386	0.384	0.371
2.5	0.716 0.745	0.731	0.718
5	1.317 1.375	1.346	1.333
10	2.240 2.397	2.319	2.306

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.

CELL CULTURE SUPERNATE/SALIVA/URINE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (ng/mL)	1.01	2.90	5.26	1.01	2.79	5.27
Standard deviation	0.042	0.173	0.344	0.079	0.188	0.326
CV (%)	4.2	6.0	6.5	7.8	6.7	6.2

SERUM/PLASMA ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (ng/mL)	1.23	3.45	6.09	1.26	3.45	6.38
Standard deviation	0.054	0.116	0.181	0.086	0.197	0.467
CV (%)	4.4	3.4	3.0	6.8	5.7	7.3

RECOVERY

The recovery of human TIMP-2 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	95	81-108%
Urine (n=4)	94	84-105%

SENSITIVITY

Sixty-eight assays were evaluated and the minimum detectable dose (MDD) of human TIMP-2 ranged from 0.004-0.064 ng/mL. The mean MDD was 0.011 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.

CALIBRATION

This immunoassay is calibrated against a highly purified CHO cell-expressed recombinant human TIMP-2 produced at R&D Systems®.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human TIMP-2 were diluted with calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Saliva* (n=4)	Urine* (n=4)
1:2	Average % of Expected	102	99	99	102	101	104
	Range (%)	101-103	87-102	93-106	93-110	97-105	101-108
1:4	Average % of Expected	105	101	101	104	101	106
	Range (%)	101-107	91-111	90-109	98-112	93-106	103-112
1:8	Average % of Expected	108	98	97	101	101	108
	Range (%)	102-112	86-106	89-102	95-113	93-103	104-115
1:16	Average % of Expected	106	———	———	———	———	112
	Range (%)	96-114	———	———	———	———	107-121

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

SAMPLE VALUES

Serum/Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human TIMP-2 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 = 60)	106	23-328	48.3
EDTA plasma (n = 37)	100	19-254	54.4
Heparin plasma (n = 36)	87	42-169	30.5
Saliva (n = 6)	16	10-22	——

Sample Type	Mean (µg/g creatinine)	Range (µg/g creatinine)	Standard Deviation (µg/g creatinine)
Urine (n=10)	4.10	2.61-6.33	1.02

Cell Culture Supernates - Human peripheral blood mononuclear cells (5 x 10^6 cells/mL) were cultured in DMEM 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 supernate were removed on days 1 and 5 and assayed for levels of human TIMP-2.

Condition	Day 1 (ng/mL)	Day 5 (ng/mL)
Unstimulated	5.13	3.87
Stimulated	2.33	1.95

SPECIFICITY

This assay recognizes recombinant and natural human TIMP-2.

The factors listed below were prepared at 50 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human TIMP-2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

MMP-1
MMP-3
MMP-7
MMP-8
MMP-9
MMP-10
MMP-13
TIMP-1
TIMP-3
TIMP-4

Recombinant mouse:

MMP-3

This assay detects approximately 50% of recombinant human TIMP-2 when complexed with recombinant human active MMP-9 in a 1:1 molar ratio.

Recombinant human MMP-2 does not interfere but does cross-react 0.002% at concentrations > 2.5 ng/mL.

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