Quantikine® ELISA

Human TIMP-4 Immunoassay

Catalog Number DTM400

For the quantitative determination of human Tissue Inhibitors of Metalloproteinases 4 (TIMP-4) concentrations in cell culture supernates, serum, plasma, and human milk.

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

MANUFACTURED AND DISTRIBUTED BY:

USA & Canada | R&D Systems, Inc.
614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
E-MAIL: info@RnDSystems.com

DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
TEL: +44 (0)1235 529449 FAX: +44 (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

China | R&D Systems China Co., Ltd.
24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

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-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs).

Among the four known members of the TIMP family, TIMP-4 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 include the binding of its C-terminal domain to the hemopexin-like domain of pro-MMP-2. Unlike TIMP-2, TIMP-4 does not promote pro-MMP-2 activation by active MMP-14 (MT1-MMP) (4, 5).

TIMP-4 mRNA is expressed at high levels in the heart, low levels in the kidney, pancreas, colon and testes (6). The plasma levels of TIMP-4 are reduced in patients with hypertrophic obstructive cardiomyopathy after alcohol septal ablation, indicating that TIMP-4 plays an important role in myocardial remodeling (7). In addition, TIMP-4 expression is regulated in several cancer types including breast, cervical, and endometrial cancer, gliomas, and choriocarcinoma (6, 8-12).

The Quantikine Human TIMP-4 Immunoassay is a 4.5 hour solid phase ELISA designed to measure TIMP-4 levels in cell culture supernates, serum, plasma, and human milk. It contains *Sf* 21-expressed recombinant human TIMP-4 and antibodies raised against the recombinant protein. This kit detects recombinant human TIMP-4 in both free and MMP-bound forms. Natural human TIMP-4 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 natural human TIMP-4.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human TIMP-4 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any TIMP-4 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human TIMP-4 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-4 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 the appropriate 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
TIMP-4 Microplate	892556	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against human TIMP-4.	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.*
Calibrator Diluent RD6-31	895323	2 vials (21 mL/vial) of a concentrated buffered protein base with preservatives.	Prepare fresh diluent for each assay. Discard the diluted diluent after use.
TIMP-4 Conjugate	892557	21 mL of a monoclonal antibody against human TIMP-4 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
TIMP-4 Standard	892558	50 ng of recombinant human TIMP-4 in a buffered protein base with preservatives; lyophilized.	
Assay Diluent RD1-89	895881	11 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.
- Test tubes for dilution of standards and samples.
- Human TIMP-4 controls (optional; available from R&D Systems).

PRECAUTIONS

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

Some components in this kit contain ProClin® 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. Please 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 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.*

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and centrifuge twice more for a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Human milk samples require a minimum 50-fold dilution. A suggested 50-fold dilution is 10 µL of sample + 490 µL of Calibrator Diluent RD6-31.

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.

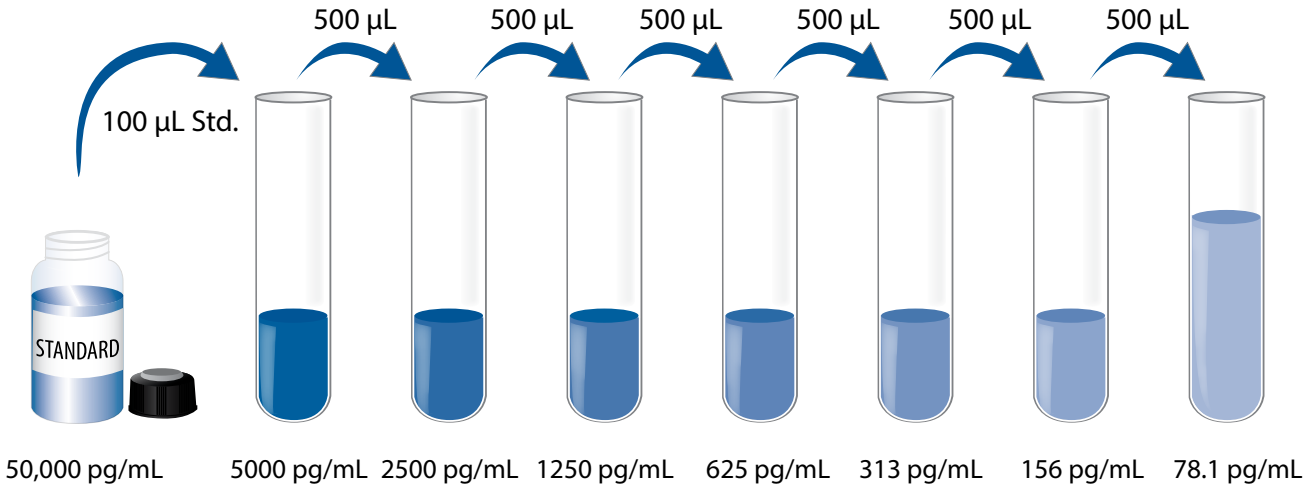
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD6-31 (1X) - Add 2 mL of Calibrator Diluent RD6-31 to 8 mL of deionized or distilled water to prepare 10 mL of Calibrator Diluent RD6-31 (1X).

Note: *Prepare only as much diluent as needed. Fresh diluent must be prepared for each assay.*

TIMP-4 Standard - Reconstitute the TIMP-4 Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 50,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD6-31 (*for serum/plasma/human milk samples*) or Calibrator Diluent RD6-31 (1X) (*for cell culture supernate samples*) into the 5000 pg/mL tube. Pipette 500 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 5000 pg/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).



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.

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 RD1-89 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.
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 TIMP-4 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours 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. **Protect from light.**
9. Add 50 mL 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.

*Human milk samples 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.).

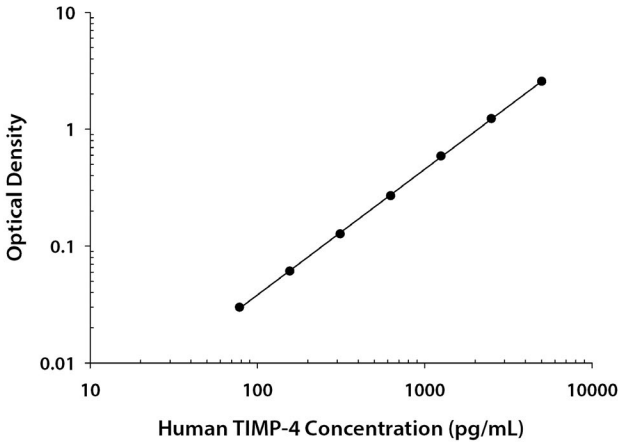
Create a standard curve by reducing the data using computer software capable of generating a log/log curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on a log/log graph. The data may be linearized by plotting the log of the TIMP-4 concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

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

TYPICAL DATA

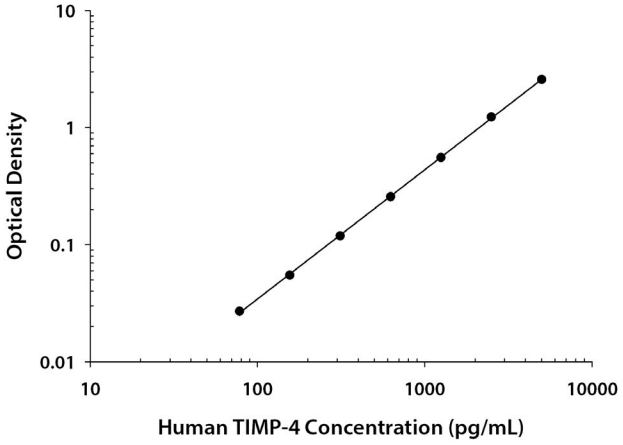
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CALIBRATOR DILUENT RD6-31



(pg/mL)	O.D.	Average	Corrected
0	0.016 0.016	0.016	—
78.1	0.045 0.046	0.046	0.030
156	0.076 0.078	0.077	0.061
312	0.141 0.144	0.143	0.127
625	0.281 0.288	0.285	0.269
1250	0.603 0.606	0.605	0.589
2500	1.240 1.252	1.246	1.230
5000	2.562 2.599	2.581	2.565

CALIBRATOR DILUENT RD6-31 (1X)



(pg/mL)	O.D.	Average	Corrected
0	0.012 0.012	0.012	—
78.1	0.038 0.039	0.039	0.027
156	0.066 0.068	0.067	0.055
312	0.129 0.132	0.131	0.119
625	0.269 0.269	0.269	0.257
1250	0.559 0.573	0.566	0.554
2500	1.235 1.249	1.242	1.230
5000	2.578 2.590	2.584	2.572

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 ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	252	793	1476	232	708	1377
Standard deviation	11.0	46.9	77.8	20.8	45.8	96.4
CV (%)	4.4	5.9	5.3	9.0	6.5	7.0

SERUM/PLASMA/HUMAN MILK ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/mL)	235	768	1516	220	712	1414
Standard deviation	13.2	21.5	70.4	20.2	48.2	87.6
CV (%)	5.6	2.8	4.6	9.2	6.8	6.2

RECOVERY

The recovery of TIMP-4 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n=4)	96	87-105%
Serum (n=4)	96	86-103%
EDTA plasma (n=4)	93	86-104%
Heparin plasma (n=4)	95	87-113%

SENSITIVITY

Eighty assays were evaluated and the minimum detectable dose (MDD) of TIMP-4 ranged from 2.14-10.0 pg/mL. The mean MDD was 4.91 pg/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.

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of TIMP-4 were serially diluted with the appropriate Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Human milk* (n=4)
1:2	Average % of Expected	101	100	101	103	101
	Range (%)	101-103	97-102	94-105	98-107	98-104
1:4	Average % of Expected	97	103	101	105	101
	Range (%)	94-100	96-107	96-104	97-109	99-102
1:8	Average % of Expected	93	107	107	111	104
	Range (%)	85-100	99-115	99-114	104-116	103-105
1:16	Average % of Expected	95	108	109	110	105
	Range (%)	90-101	102-116	101-115	102-118	99-110

CALIBRATION

This immunoassay is calibrated against a highly purified *Sf* 21-expressed recombinant human TIMP-4 produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of TIMP-4 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=35)	1054	460-2661	482
EDTA plasma (n=35)	1044	516-2476	416
Heparin plasma (n=35)	1133	664-2328	417
Human milk (n=4)	57,800	25,700-111,000	43,800

Cell Culture Supernates:
Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in RPMI supplemented with 10% fetal calf serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernate were removed and assayed for levels of natural TIMP-4. No detectable levels were observed.

MDA-MB-231 human breast cancer cells were cultured in Leibovitz L-15 media with 10% fetal calf serum and 2 mM L-glutamine and incubated at 37 °C. An aliquot of the cell culture supernate was removed, assayed for natural TIMP-4, and measured 191 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant human TIMP-4.

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-4 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:		Recombinant mouse:	Recombinant rat:
ADAM8	MMP-8	ADAM9	TIMP-1
ADAM10	MMP-9	ADAM10	
ADAM15	MMP-10	ADAM15	
ADAM17	MMP-12	ADAM33	
ADAMSL1.2	MMP-13	MMP-2	
ADAMTS1	MMP-14	MMP-3	
MMP-1	MMP-16	MMP-9	
MMP-2	TIMP-1	TIMP-1	
MMP-3	TIMP-2		
MMP-7	TIMP-3		

This kit detects recombinant human TIMP-4 in both free and MMP-bound forms.

REFERENCES

1. Nagase, H. and J.F. Woessner, Jr. (1999) J. Biol. Chem. **274**:21491.
2. Parks, W.C. and R.P. Mecham (1998) Matrix Metalloproteinases, Academic Press, San Diego.
3. Brew, K. *et al.* (2000) Biochim Biophys. Acta **1477**:267.
4. Hernandez-Barrantes, S. *et al.* (2001) Biochem. Biophys. Res. Comm. **281**:126.
5. Kai, H.S-T. *et al.* (2002) J. Biol. Chem. **277**:48696.
6. Greene, J. *et al.* (1996) J. Biol. Chem. **271**:30375.
7. Stroud, R.E. *et al.* (2005) J. Card. Fail. **11**:124.
8. Zhao, Y-G. *et al.* (2004) Cancer Res. **64**:590.
9. Lizarraga, F. *et al.* (2005) Anticancer Res. **25**:623.
10. Tunuguntla, R. *et al.* (2003) Gynecol. Oncol. **89**:453.
11. Groft, L.L. *et al.* (2001) Br. J. Cancer **85**:55.
12. Zhang, J. *et al.* (2002) Mol. Hum. Reprod. **8**:659.