

INSTRUCTIONS FOR USE

TIMP-1 ELISA

Item No. 06489973

The logo for WILEX, featuring the word "WILEX" in a bold, black, sans-serif font. A small red triangle is positioned above the letter "X".

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OncogeneScience

Manufactured by:
WILEX Inc.
Cambridge, MA 02140 USA

Intended Use

The TIMP-1 ELISA is an enzyme-linked immunoassay for the quantitation of total (free and complexed) tissue inhibitor of metalloproteinase-1 (TIMP-1) in human serum and plasma.

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Method Principle	Solid Phase Sandwich ELISA
Analytical Range	0 nanograms/mL (ng/mL) to 1000 ng/mL
Specimen Types	Human serum and plasma
Sample Test Volume	100.0 microliters of diluted sample per well
Sensitivity	0.068 ng/mL

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Background

Tissue inhibitor of metalloproteinase-1 (TIMP-1) is a 28-kDa protein present in most human tissues and body fluids that inhibits matrix metalloproteinases (MMPs), stimulates cell growth, and prevents apoptosis [1,2]. Numerous studies have examined the level of TIMP-1 in serum and plasma in association with disease.

Matrix metalloproteinases degrade proteins in the extracellular matrix (ECM), playing an important role in development, morphogenesis, and wound healing [3]. Their unregulated activity is implicated in a wide variety of diseases from atherosclerosis to cancer. MMPs are regulated by binding to a family of homologous proteins known as tissue inhibitors of metalloproteinases (TIMPs) [2]. TIMPs have two domains, an N-terminal inhibitory domain and a C-terminal domain, which can influence their binding affinity [4]. TIMPs and MMPs form 1:1 enzyme inhibitor complexes. The correct balance between TIMPs and MMPs is critical for ECM metabolism.

Investigators examined the level of TIMP-1 in different cancers. Holten-Anderson, et al., reported that total levels of TIMP-1 were significantly higher in patients with colonic and rectal cancer than in healthy patients or patients with inflammatory bowel disease [5]. Pellegrini, et al., compared the serum levels of TIMP-1 to the serum levels of carcinoembryonic antigens (sCEA) in patients with colorectal cancer. They found that simultaneous measurement of the two could be used as a marker of disease progression from the pre-invasive to the invasive phase [6]. Researchers have shown that expression levels of TIMP-1 in the plasma of patients with ovarian carcinoma were found to be significantly higher than that in normal patients [7]. Jung, et al.,

analyzed plasma concentrations of TIMP-1 in healthy males, in patients with benign prostatic hypertrophy (BPH), and in patients with prostate cancer (PCa) with and without metastatic disease. They found that TIMP-1 concentrations were significantly higher in PCa with metastases compared with controls, BPH, and PCa patients without metastases [8]. Susskind, et al., found elevated levels of TIMP-1 in the plasma of patients with lung and breast cancer [9].

The serum levels of TIMP-1 have been investigated as a marker of chronic liver disease. Serum TIMP-1 levels in patients with chronic liver disease showed significant correlations with the stage of disease determined by histomorphological evaluation of liver biopsies [10]. Nie, et al., compared the levels of TIMP-1 in the liver and serum of patients with cirrhosis. Immunohistochemistry showed that TIMP-1 was present in all diseased livers and not present in normal livers. This correlated with the finding that TIMP-1 was elevated in 74% of serum samples from patients with diseased livers [10].

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The levels of TIMP-1 have been evaluated in a variety of other diseases. The ratio between metalloproteinases and their inhibitors has been investigated in association with multiple sclerosis. A high serum ratio of MMP-9/TIMP-1 is linked to the activity phase of the disease [11]. Jinnin, et al., found that the serum levels of TIMP-1 in patients with mixed connective tissue disease were significantly higher than those in healthy patients [12].

The TIMP-1 ELISA is designed to provide the investigator with a convenient, accurate, and reproducible method to determine TIMP-1 levels in human serum or plasma. Clinical research, using the TIMP-1 ELISA, will help to better define the role of TIMP-1 in cancer and other diseases.

Principle of the Assay

The TIMP-1 ELISA is a sandwich-type immunoassay that uses a mouse monoclonal Capture Antibody and an alkaline phosphatase conjugated mouse monoclonal antibody as detector. This combination of capture and detector reagents allows detection of free TIMP-1 and TIMP-1 complexed to metalloproteinases such as MMP-9 [13]. The Capture Antibody has been immobilized on the interior surface of the microtiter plate wells. To perform the test, an appropriate volume of specimen is incubated in the wells to allow binding of the antigen by the Capture Antibody. The immobilized antigen is then exposed to the alkaline phosphatase-labeled Detector Antibody (Conjugate). Addition of Substrate to the wells allows the catalysis of a chromogen into a colored product, the intensity of which is proportional to the amount of TIMP-1 that is bound to the plate.

Standards are provided in the kit that allow accurate, quantitative determinations of TIMP-1 in suitable samples. Using a microtiter plate reader, one can measure simultaneously the absorbance of the colored product in the Standards and sample wells. Correlating the absorbance values of samples with the Standards allows the investigator to determine the levels of TIMP-1 in a sample. Samples may be assigned a quantitative value of TIMP-1 in ng/mL of serum or plasma.

For instructions, see the Detailed Protocol and Evaluation of Results sections of this booklet.

Summary of Procedure

Steps	Incubations
1. Add samples and Standards to wells	2 hours, RT*
2. Wash	
3. Add Conjugate to wells	30 minutes, RT*
4. Wash	
5. Add Substrate to wells	25 minutes, RT* in the dark
6. Add Stop Solution to wells	
7. Read plate at 405 nm	

*Room temperature (20–27°C)

Materials Provided

The following components are supplied:

Microtiter plate—One (1) precoated microtiter plate supplied ready to use, with 96 wells (12 strips of eight) in a zip-lock bag with a desiccant pack. Wells are coated with an anti-human TIMP-1 monoclonal antibody.

TIMP-1 Standards—Six (6) separate vials containing purified Lyophilized TIMP-1. Reconstitute each vial of Standard prior to use with 1.0 mL of the Filtered RO Water included in this kit. Do NOT dilute Standards. After reconstituting, store at $\leq -18^{\circ}\text{C}$. See Detailed Protocol for instructions.

Standards are labeled with values that are 50-fold greater than the actual vial dosage. Assigning these label values to a standard curve obviates the need to correct the reported sample recoveries for a 1:50 diluted sample (2% serum in buffer). See Evaluation of Results. Standards contain 0.09% sodium azide.

Standard#	Concentration (ng/mL)
L6	1000.0
L5	500.0
L4	330.0
L3	220.0
L2	44.0
L1	0.0

Filtered RO Water—One (1) 7-mL bottle containing Filtered RO Water.

Sample Diluent—One (1) 100-mL bottle containing BSA, mouse IgG, and 0.09% sodium azide.

Conjugate—One (1) 12-mL bottle containing alkaline phosphatase-labeled anti-TIMP-1 antibody.

Substrate—One (1) 12-mL bottle containing pNPP Substrate.

Stop Solution—One (1) 12-mL bottle containing EDTA.

Platwash Concentrate—One (1) 100-mL bottle. Dilute one (1) part concentrate in 19 parts high-quality deionized water prior to use.

Materials Required but Not Provided

- Pipettors: 2–20 μL , 20–200 μL , and 200–1000 μL precision pipettors with disposable tips
- Precision manual or automated repeating pipettor for 5- and 10-mL pipets
- Disposable 5- and 10-mL pipets
- Wash bottle, multichannel dispenser, or automated 96-well microtiter plate washer for plate washing
- Microcentrifuge and tubes for sample preparation
- 500- or 1000-mL graduated cylinder
- High-quality deionized water

- 12 x 75 mm culture tubes for sample preparation
- Vortex mixer
- Reagent reservoirs
- Plastic wrap or adhesive plate sealers
- Water bath
- Microtiter plate reader capable of measuring absorbance at a wavelength of 405 nm
- Liquid household bleach for inactivating clinical specimens and decontamination of plate washer
- Disposable paper towels
- Ice bucket and ice

TIMP-1 ELISA Controls—Controls have been developed to provide customers with control material for quality monitoring of day-to-day assay performance. TIMP-1 ELISA Controls consist of Lyophilized TIMP-1. Refer to TIMP-1 ELISA Controls, Item No. 06489981, when ordering. Controls are supplied lyophilized and are reconstituted prior to use with 0.5 mL of the Filtered RO Water included in the Controls kit. Store dry Lyophilized TIMP-1 ELISA Controls at 2–8°C. Liquid Reconstituted Controls must be stored at $\leq -18^{\circ}\text{C}$. Protect Controls from light. Thaw frozen liquid Reconstituted Controls on ice prior to use.

Precautions and Recommendations

- Store components at 2–8°C, except for Platewash Concentrate and liquid Reconstituted Standards. Store Platewash Concentrate at room temperature. Store Reconstituted Standards at $\leq -18^{\circ}\text{C}$. Do not expose reagents to excessive light.
- Do not use kit components beyond the indicated kit expiration date.
- Use only the microtiter wells provided with the kit.
- Do not mix reagents from different kits.
- The buffers and reagents used in this kit contain sodium azide as a preservative. Care should be taken to avoid direct contact with this reagent.
- Do not mouth pipet or ingest any of the reagents.
- Do not smoke, eat, or drink when performing the assay or in areas where samples or reagents are handled.
- Human samples may be contaminated with infectious agents. Do not ingest, expose to open wounds, or breathe aerosols. Wear protective gloves and dispose of biological samples properly.

Sample Preparation

Suitable samples for analysis by the TIMP-1 ELISA include human plasma treated with heparin, citrate, or EDTA, and human serum. Due to possible interfering factors, special care must be taken in the preparation and assay of human serum and plasma. Predilute serum and plasma at 1:50 as described. Remove any flocculent material from samples by microcentrifugation prior to dilution.

SERUM OR PLASMA

The initial concentration of the serum or plasma specimen to be examined should not exceed a concentration of 2% (a 1:50 dilution of specimen in Sample Diluent). For example, 0.020 mL of sample may be diluted into 0.980 mL of Sample Diluent and 100 μ L of this preparation added to the microtiter plate wells.

Detailed Protocol

RECOMMENDED PROCEDURES

1. Addition of reagents must be in the order specified.
2. Preparation, handling, and storage of Standards:
 - a. Prior to opening the vials, ensure the dried material is located at the bottom of each vial. Gently tap the vial if necessary to reposition the material.
 - b. Reconstitute the dry Lyophilized Standards by careful and accurate addition of 1 mL of the Filtered RO Water included with this kit.

- c. Replace the rubber stopper, gently swirl the vials, and then allow them to sit for 5 minutes. Gently invert the vials to ensure that all of the lyophilized material is dissolved and to ensure a uniform concentration throughout the solution. When the Lyophilized Standards are completely reconstituted, they are ready to use.
- d. Replace the outer black cap on the vials on top of the inner rubber stopper before storing the liquid Reconstituted Standards at $\leq -18^{\circ}\text{C}$. Both the outer cap and inner stopper are required in order to form a tight seal on the vial.

IMPORTANT: Minimize the time liquid Reconstituted Standards are kept at room temperature and store in the box provided at $\leq -18^{\circ}\text{C}$ immediately after each use.

- e. All six Standards and the test specimens should be run in duplicate. Change tips during this process. Avoid carryover of one Standard into another.
 - f. Thaw frozen liquid Reconstituted Standards on ice and **minimize** their time at room temperature. The thawed Reconstituted Standards must be stored at $\leq -18^{\circ}\text{C}$ immediately after use.
3. Equilibrate all reagents, **with the exception of Reconstituted Standards** (see above), to room temperature ($20\text{--}27^{\circ}\text{C}$) for at least 30 minutes prior to use.
 4. CAUTION: When inverting the microtiter plate to decant or blot, press the side tabs of the frame inward to prevent the strips from falling out. Fill the open portion of the frame with uncoated or used strips when 96-port mechanical washers are used.

5. Preparation of Platewash
 - a. If the Platewash Concentrate is cold, allow it to reach room temperature (20–27°C) before use (about 45 minutes). Make sure all crystals are dissolved. If necessary, warm at 37°C and stir.
 - b. Dilute one (1) volume of Platewash Concentrate with 19 volumes of distilled or deionized water. Mix well. This solution is Platewash. The total volume required will depend on the washing method/instrument used. Approximately 1 L of this solution is required to prime an automated washer and run one microtiter plate; about 700 mL is required for each microtiter plate when manual washing is performed.
 - c. Platewash must be freshly prepared each day. Do not store Platewash.
6. Microtiter plate washing may be automated, semi-automated, or manual, but must be carried out with care to ensure optimal performance of the assay. Plate washing equipment must be properly adjusted, cleaned, and maintained. Whichever method is used, the solution used to wash plates is Platewash.
 - a. Automatic Microtiter Plate Washer—Set the fill volume to 300 μ L/well. Prime the instrument with Platewash. Use two 3-cycle washes. After the initial 3-cycle wash, rotate the plate 180° and repeat.

- b. Manual Microtiter Plate Washer—Wash six times, using 300 μL per well per wash. Fill the entire plate, then aspirate in the same order.
- c. Hand-Held Syringe—Wash six times, using 300 μL per well per wash. Blot the plate upside-down between washes.

After the final wash, invert the microtiter plate and tap it on an absorbent surface to remove excess liquid. Wells should not be completely dry. Residual liquid protects the bound reagents from desiccation. This is particularly important for maintaining enzyme activity.

7. The transfer of samples and Standards from tubes to the microtiter wells can be greatly simplified by using semi-automation. The hand-held expandable and programmable 8-place pipet, Impact EXP[®] (Matrix Technologies Corp., Hudson, NH), is ideal for this purpose.

Assay Procedure

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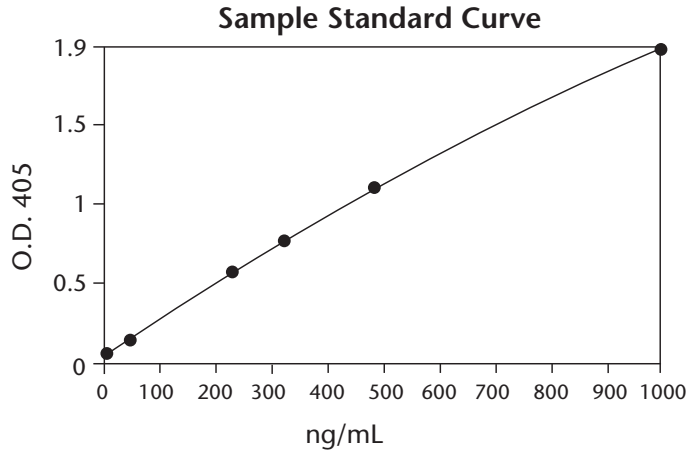
The TIMP-1 ELISA is provided with removable strips of wells so the assay can be carried out on multiple occasions. A standard curve must be included in each separate assay. Both the Standards and samples should be assayed in duplicate. Disposable pipet tips and clean reagent troughs must be used for all transfers to avoid cross-contamination of reagents and samples.

IMPORTANT: Warm all samples and kit reagents, with the exception of liquid Reconstituted Standards (for reconstitution, see Detailed Protocol), to room temperature before addition to assay plate wells. Frozen Liquid Reconstituted Standards should be thawed on ice.

1. Cut the foil pouch between the notches at the zip-lock end, break the zip-lock seal, and remove the plate from the foil pouch. Select the number of 8-well strips needed. Remove unused strips from the frame and return to the pouch. Seal the zip lock and store at 2–8°C. (Save the frame for future assays.)
2. Dilute specimens and controls 1:50 using the Sample Diluent. Reconstitute or thaw Standards. Do NOT dilute Standards. See Detailed Protocol.
3. Add diluted samples and each of the six TIMP-1 Reconstituted Standards (L1 to L6) in duplicate by pipetting 100 µL into appropriate wells using clean pipet tips for each sample and Standard. Add Standard L1 to one additional well to be used for determination of Substrate blank. **Store the liquid Reconstituted Standards in the box provided at $\leq -18^{\circ}\text{C}$ immediately after use.**
4. Cover wells with clean plastic wrap or plate sealer. Incubate plate for 2 hours at room temperature (20–27°C).
5. Prepare a working solution (1X) of Platewash. Add one (1) part Platewash Concentrate to 19 parts of deionized water. Mix well.
6. Carefully remove the plastic wrap or plate sealer. Wash wells for six cycles using 300 µL per well of Platewash. (Wash for three cycles, rotate the plate 180°, and wash for three more cycles.) After the final wash, invert the plate and tap it on an absorbant surface to remove any excess liquid. CAUTION: Do not allow plates to dry out. Proceed immediately to Step 7.

7. Pipet 100 μL of Conjugate into all wells **except the Substrate blank well**. Cover the wells with a fresh piece of plastic wrap or plate sealer. Incubate plate at room temperature (20–27°C) for 30 minutes.
8. Wash wells as in Step 6. CAUTION: Do not allow plates to dry out. Proceed immediately to Step 9.
9. Pipet 100 μL of Substrate into all wells and cover the plate with plastic wrap or plate sealer. Incubate plate at room temperature (20–27°C) for 25 minutes in the dark.
10. Pipet 100 μL of Stop Solution into all wells.
11. Measure absorbance in each well using a spectrophotometric plate reader at a wavelength of 405 nm. Wells should be read within 30 minutes of adding the Stop Solution.

Figure 1



Evaluation of Results

1. Average the absorbance values for each Standard and all sample dilutions to obtain the mean absorbencies.
2. Do not assign “blank” wells using software. This will subtract the average blank readings from all other wells. The Substrate blank should be labeled as a sample so no subtraction occurs. It is useful for quality control and troubleshooting purposes to be able to inspect the absorbance values reported for the Substrate blank without adjustments applied to the raw data.
3. Design a standard curve (**Figure 1**, p. 17) by plotting the mean absorbance for each Standard on the y-axis versus the concentration of the Standards on the x-axis. There are a variety of microtiter plate reader software packages available for analysis of microtiter plate data (SoftMax Pro™, Molecular Devices Corporation, Sunnyvale, CA; KC4™, Bio-Tek Instruments, Inc., Winooski, VT) that simplify this process. The standard curve concentrations are assigned the value indicated on the vial and in the Materials Provided section of this booklet. Use a quadratic curve fit algorithm (second order polynomial).

4. Results for samples and Controls are expressed in ng/mL by reading directly from the standard curve concentrations. For convenience, no mathematical dilution correction is needed for 1:50 diluted samples since the absolute concentration in the Standard preparations is at 2% of the labeled dosage (i.e., they have been prediluted at 1:50).
5. For samples that give absorbance (OD) values exceeding the range of the standard curve, subsequent assay at greater dilutions will be necessary. Any such sample result will require correcting the value obtained from the assay for any dilution beyond 1:50.

Example:

Sample	Dilution Correction Factor (multiply reported result by)
1:100	2
1:200	4
1:400	8

Sample Values

The levels listed should be used as a guideline only. The determination of normal ranges should be carried out by each laboratory using appropriate samples.

TIMP-1 LEVELS IN NORMAL HUMAN SAMPLES (ng/mL)

	n	Range	Mean	Mean + 2SD	95% fall below:
EDTA Plasma	99	100-305	175	257	263
Serum	198	68-802	197	420	433

TIMP-1 LEVELS IN MATCHED HUMAN CANCER SERUM AND EDTA PLASMA SAMPLES

Sample	Level in Serum, ng/mL	Level in EDTA Plasma, ng/mL
1	380	257
2	530	357
3	627	275
4	224	226
5	895	670
6	589	319
7	708	305
8	908	650

Assay Characteristics

ANALYTICAL SENSITIVITY

Minimal detectable concentration of analyte was determined by repeated measurement of a zero dose sample (Standard Level 1) and calculation of mean + 2 standard deviations. The TIMP-1 ELISA is capable of detecting 0.068 ng/mL TIMP-1 when using the absolute values of the Standards in the kit. As a convenience to the customer, Standards are labeled at a 50-fold higher concentration than the absolute dose. And, since samples are diluted at 1:50 prior to assay, the practical limit of analytical sensitivity for sample analysis is 50 fold at 3.4 ng/mL.

SPECIFICITY

TIMP-2, TIMP-3, and TIMP-4 are isoforms of TIMP-1. The TIMP-1 ELISA was challenged with high levels of each isoform. They produced no signal in the TIMP-1 ELISA, indicating that there is no cross-reactivity with TIMP-2, TIMP-3, or TIMP-4.

PRECISION

1. Inter-Assay

Normal human serum spiked with TIMP-1 at three different concentrations was tested in three assays by three different operators with three replicates per test point. Between assay variability was below 9.0%.

Sample	High	Medium	Low
n	9	9	9
Mean (ng/mL)	587.0	445.8	229.3
% C.V.	5.0	8.3	8.9

2. Intra-Assay

Normal human plasma was spiked with TIMP-1 at three different concentrations with eight replicates per test point. Within assay variability was below 7.0%.

Sample	High	Medium	Low
n	8	8	8
Mean (ng/mL)	666.0	439.7	74.0
% C.V.	6.5	4.6	6.0

PARALLELISM

Parallelism tests the ability of the TIMP-1 ELISA to report accurate recoveries for serial dilutions of samples. Serum and plasma samples were initially diluted 1:50, subsequent two-fold dilutions were made, and all dilutions were tested in the TIMP-1 ELISA. Recoveries (ng/mL) for each dilution were calculated by correcting with the appropriate dilution factor. Results show that plasma and serum samples diluted in Sample Diluent result in accurate recovery of TIMP-1.

Dilution	Plasma Sample		Serum Sample	
	OD	ng/mL	OD	ng/mL
1:50	0.95	430.0	1.62	768.6
1:100	0.53	418.6	0.89	692.7
1:200	0.30	397.3	0.53	733.7

RECOVERY

TIMP-1 was spiked into normal human serum or Sample Diluent at high, medium, and low concentrations and each was tested in the TIMP-1 ELISA. Results showed that the recovery of TIMP-1 at all levels was at least 95%.

Troubleshooting

A. ASSAY DOES NOT DEVELOP COLOR OR ODs ARE LOW

- Plate allowed to dry out after Conjugate step.
- Step(s) omitted or in wrong sequence.
- Room temperature during incubations fell below 20°C.
- Substrate was not warmed to room temperature (20–27°C) prior to addition to wells.

B. HIGH BACKGROUND SIGNAL

- Insufficient washing between steps.
- The Substrate blank well should read ≤ 0.11 absorbance units. Zero Standard should read ≤ 0.15 absorbance units. Higher readings indicate deterioration of Substrate or exposure of Substrate to light before or during the incubation step.
- Be certain the plate is read at the correct wavelength.

C. POOR DUPLICATES

- Insufficient washing, especially when accompanied by high background. Take special care when washing plates by hand, or have automatic washer serviced.

- Sporadic high signals may indicate contamination of Substrate by Conjugate. Be sure to use a fresh piece of plastic wrap or adhesive plate sealer for this step. Residual droplets of Conjugate on re-used plastic wrap or adhesive plate sealer may lead to false positive signals.
- Generating bubbles in wells on addition of reagents. Use care in pipetting.
- Splashing of reagents between wells will lead to erroneous results. Avoid jarring the plate.

Reagent Stability and Storage

All of the reagents included with the TIMP-1 ELISA have been tested for stability. Reagents should not be used beyond the stated expiration date. Kit reagents, with the exception of Reconstituted Standards and Platewash, should be stored at 2–8°C. Coated assay plates should be stored in the original foil bag sealed by the zip lock and containing a desiccant pack. Reconstituted Standards must be stored at $\leq -18^{\circ}\text{C}$ for up to 30 days. Minimize the time that Reconstituted Standards are kept at room temperature. Platewash can be stored at room temperature (20–27°C).

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Notes:



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