

Human TIMP-4

(Metalloproteinase Inhibitor-4)

Pre-Coated ELISA Kit

Catalog No: 90-2127

1 × 96 well Format (96 tests)

Detection Range: 78 – 5000 pg/ml

Sensitivity: < 46.875 pg/ml

This immunoassay kit allows for the in vitro quantitative determination of Human TIMP-4 concentrations in serum, plasma and other biological fluids.

This kit is for Research Use Only. Not for use in diagnostic/therapeutics procedures.

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I. BACKGROUND

Tissue inhibitors of matrix metalloproteinases (TIMPs) comprise a family of four members, of which TIMP-4 is characterized by its restriction to cardiovascular structures. TIMP-4 is endogenously one of the key modulators of matrix metalloproteinase 9 (MMP9) and instigates contractility and helps in differentiation of cardiac progenitor cells. TIMP-4 is expressed highly in heart and found dysregulated in human cardiovascular diseases. It controls extracellular matrix remodeling by inhibiting matrix metalloproteinases (MMPs) and is implicated in processes including cell proliferation, apoptosis, and angiogenesis. It modulates multiple signaling pathways relevant in cancer in MCF7 cells, including the ER-alpha cascade. TIMP-4 is present in significant amounts in human atherosclerotic coronary artery lesions, but its relations with the early pathogenesis of atherosclerotic changes have not been clarified.

II. OVERVIEW

This assay employs an antibody specific for Anti-human TIMP-4 coated on a 96-well plate. Standards and samples are pipetted into the wells and TIMP-4 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human TIMP-4 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of TIMP-4 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

III. ADVANTAGES

Multiple samples can be analyzed in a low volume, high-throughput format. Full analysis can be complete in 2 hours.

IV. STORAGE

Kit can be stored in 4°C, if you are using within a week.

If you are using within 6 months, lyophilized standard can be stored in -20°C and other components at 4°C.

Kit Components

| Item | Specifications | Storage |
|--|----------------|----------------------------|
| 96 well Strip ELISA Plate | 8 × 12 well | 4°C |
| Lyophilized Standard | 2 vials | -20°C |
| Sample and Standard Dilution Buffer | 20 ml | 4°C |
| Biotinylated Detection Antibody for h TIMP-4 | 120 µl | 4°C (Protected from light) |
| Antibody Dilution Buffer | 10 ml | 4°C |
| HRP Conjugated Streptavidin | 120 µl | 4°C (Protected from light) |
| Streptavidin Dilution Buffer | 10 ml | 4°C |
| TMB Substrate | 10 ml | 4°C |
| Stop Solution | 10 ml | 4°C |
| 25X Wash Buffer | 30 ml | 4°C |
| Plate Sealer | 5 pieces | |
| Product Manual | 1 | |

Material Required, (Not Supplied)

Microplate Reader

37°C Incubator

Plate Reader

Multi Chanel Pipette and disposable tips

Eppendorf Tubes

Deionized Water

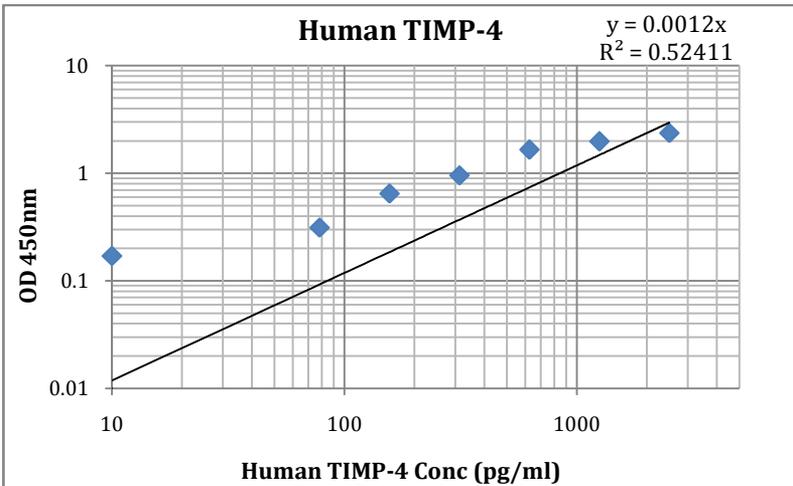
V. PRECAUTIONS FOR USE

1. To inspect the validity of experiment operation and the appropriateness of sample dilution proportion, pilot experiment using standards and a small number of samples is recommended.
2. After opening and before using, keep plate dry.

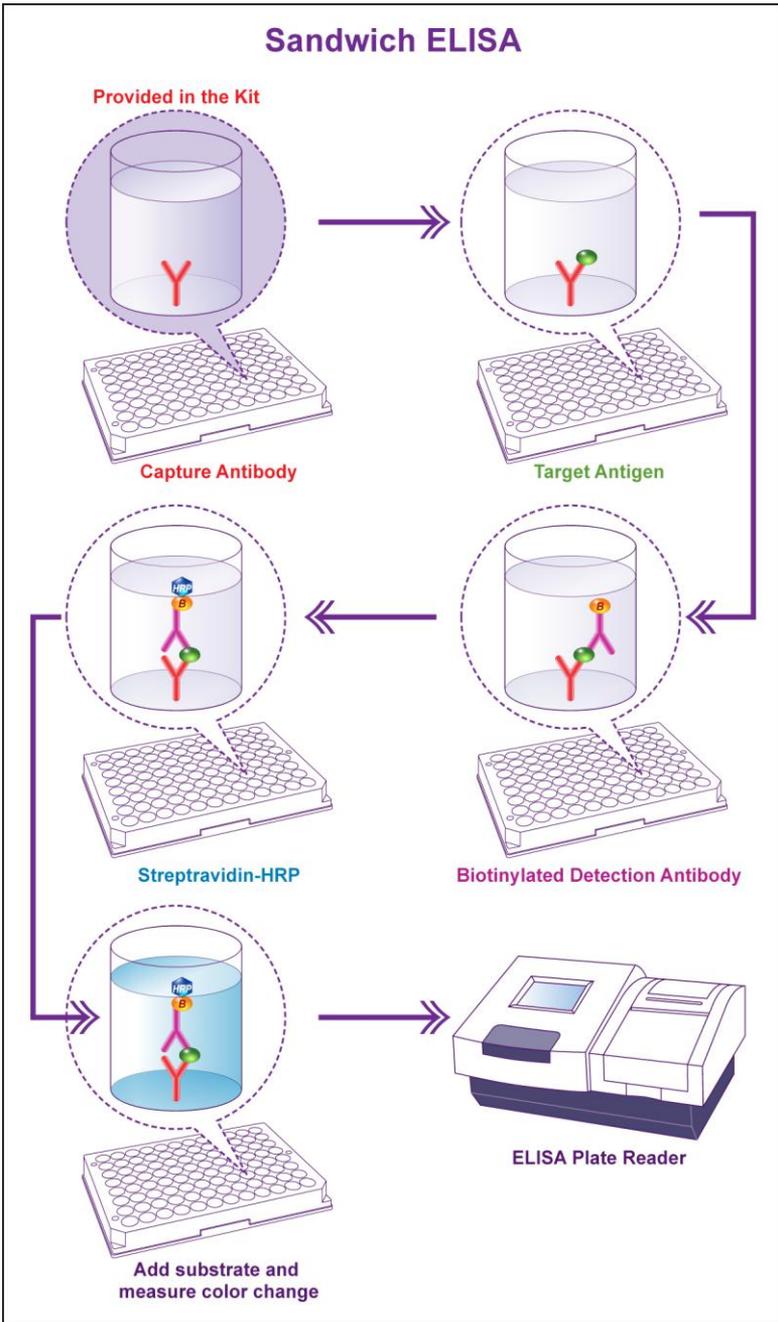
3. Before using the Kit, spin tubes and bring down all components to the bottom of tubes.
4. Storage TMB reagents avoid light.
5. Washing process is very important, not fully wash easily cause a false positive.
6. Duplicate well assay is recommended for both standard and sample testing.
7. Don't let Micro plate dry at the assay, for dry plate will inactivate active components on plate.
8. Don't reuse tips and tubes to avoid cross contamination.
9. Avoid using the reagents from different batches together.

VI. STANDARD CURVE

Human TIMP-4 Standard Curve is shown below.



| | | | | | | | | | |
|---|---------|-------|-------|------|-------|-------|-------|-------|-------|
| X | pg/ml | 5000 | 2500 | 1250 | 625 | 312.5 | 156.3 | 78.13 | 0 |
| Y | O.D.450 | 2.367 | 1.978 | 1.66 | 0.958 | 0.653 | 0.317 | 0.174 | 0.004 |



VII. REAGENT PREPARATION AND STORAGE

Included buffers and reagents are optimized for use with this kit. Substitution with other reagents is not recommended and may not give optimal results.

1. **Prepare Standard Curve:** One hour before the experiment.
 - a. Quick spin down one vial of lyophilized standard. (**DO NOT dilute standard directly on the plate**). Add 1ml of sample/standard dilution buffer into one of the standard tube. Incubate at room temp. for 10 min. Mix thoroughly by vortex. Stock Standard concentration is 5000 pg/ml.
 - b. Label 6 eppendorf tubes with 2500 pg /ml, 1250 pg/ml, 625 pg/ml, 312.5pg/ml, 156.25 pg/ml, 78.125 pg/ml respectively. Add 0.3 ml of sample/standard dilution buffer into each tube. Add 0.3 ml of stock standard (5000 pg/ml) into 1st tube and mix thoroughly. Transfer 0.3 ml from 1st tube to 2nd tube and mix thoroughly. Transfer 0.3 ml from 2nd tube to 3rd tube mix thoroughly, and so on.

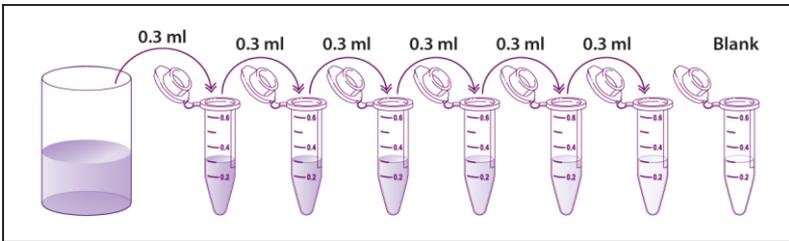


Fig-1: Dilution tubes

Note: Standard Solutions are best used within 2 hrs. Standard solution should be stored at 4°C for up to 12 hrs. or store at -20°C for up to 48 hrs. Avoid repeated freeze-thaw.

2. **Sample Preparation and storage:** Test samples should be collected, analyze immediately (within 2 hrs.) or aliquot and store at -20°C for long term. Avoid multiple freeze-thaw cycles.
 - a. **Cell culture supernatants:** Centrifuge to remove precipitate, analyze immediately or aliquot and store at -20°C.

- b. Serum:** Coagulate the serum at room temp about 1 hr. Centrifuge approximately $1000 \times g$ for 15 min. Analyze serum immediately or aliquot and store at -20°C .
- c. Plasma:** Collect plasma with heparin or EDTA as the anti-coagulant. Centrifuge for 15 min at $2-8^{\circ}\text{C}$ at $1500 \times g$ within 30 min of collection. For eliminating the platelet effect, suggesting that further centrifugation for 10 min at $2-8^{\circ}\text{C}$ at $10,000 \times g$. Analyze immediately or aliquot and store frozen at -20°C .
- d. Tissue Homogenates:** For general information, hemolytic blood may affect the results, you should rinse the tissues with ice cold PBS (0.01M, pH 7.4) to remove excess blood thoroughly. Tissue pieces should be weighed and then minced to small pieces. This will be homogenized in PBS in a cold glass homogenizer. (*Volume depends on the weight of the tissue, 1gram of tissue requires 9 ml of ice cold PBS with protease inhibitor*). To further break the cells, you can sonicate the suspension with an ultrasonic cell disrupter or subject it to freeze- thaw cycle. Homogenates are then centrifuged for 5 min. at $5000 \times g$ to get the supernatant.

Note: *Samples to be used within 5 days may be store at 4°C , otherwise sample should be stored at -20°C (< 1 month) or -80°C (<2 months) to avoid loss of bioactivity and contamination. Hemolyzed samples are not suitable for use in this Assay.*

- e.** End user should estimate the concentration of the target protein in the test samples first, then select proper dilution factor to make the diluted target protein concentration falls the optimal detection range of the kit. Dilute the samples with the provided dilution buffer. Several trials may be necessary in practice. The test sample should be well mixed with the dilution buffer. Standard curve and sample should be made before the experiment.

High target protein concentration 50-500 ng/ml: Dilute 1:100 (add $1 \mu\text{l}$ of sample into $99 \mu\text{l}$ of sample/standard dilution buffer).

Medium target protein concentration 5-50 ng/ml: Dilute 1:10 (add 10 μ l of sample into 90 μ l of sample/standard dilution buffer).

Low target protein concentration 0.078-5 ng/ml: Dilute 1:2 (add 50 μ l of sample into 50 μ l of sample/standard dilution buffer).

Very low target protein concentration < 0.078 ng/ml: Do not dilute, use 100 μ l of sample or 1:2 dilution.

- 3. Preparation of Biotin detection antibody working solution:** Prepare within one hour before the experiment. Calculate total volume working solution required. (0.1 ml/well \times number of wells. Add 100-200 μ l extra).

Dilute Biotin detection antibody with antibody dilution buffer at 1:100 and mix thoroughly. (*i.e.* add 1 μ l of Biotin conjugated detection antibody into 99 μ l of antibody dilution buffer).

- 4. Preparation of HRP-Streptavidin Conjugate (SABC) working solution:** Prepare within 30 min before the experiment. Calculate total volume working solution required. (0.1 ml/well \times number of wells. Add 100- 200 μ l extra).

Dilute SABC with SABC dilution buffer at 1:100 and mix thoroughly. (*i.e.* add 1 μ l of SABC into 99 μ l of SABC dilution buffer).

- 5. Preparation of 1 X Wash buffer:** Prepare 1 X Wash buffer by diluting 25X Wash buffer in sterile water. Diluted Wash buffer may be stored at 4°C, however we recommend preparing fresh 1X wash buffer for each experiment.

For example: 10 ml of 25X Wash buffer in 240 ml of sterile water.

VIII. ASSAY PROCEDURE

Before starting the experiment, equilibrate the SABC working solution and TMB substrate for at least 30 min at room temp. When diluting samples and reagents, they should be mixed completely and evenly. It is recommended to plot a standard curve for each test.

** If not all microtiter plate strips will be used, remove the excess strips by pressing up from underneath each strip. Place excess strips back in the foil pouch with the included desiccant pack and reseal.*

1. Set standard, test sample and blank (control zero) wells on the pre-coated plate and then record their position. It is recommended to measure each standard and sample in duplicate. **Note:** Wash plate twice before adding standard, sample and blank into the well.
2. Add 0.1 ml of standard (5000 pg/ml, 2500 pg/ml, 1250 pg/ml, 625 pg/ml, 312 pg/ml, 156.25 pg/ml, 78.125 pg/ml, control zero dilution buffer) into standard well.
3. Add 0.1 ml of diluted samples into test sample wells.
4. Seal plate with a cover and incubate at 37°C for 90 min.
5. Remove the cover and discard samples and standard solution by tapping plate on an absorbent paper. **Note:** DO NOT let the wells completely dry any time. DO NOT wash plate.
6. Add 0.1 ml of Biotin-detection antibody working solution into the above wells (Standards, control zero and samples).
7. Seal plate with cover and incubate at 37°C for 60 min.
8. Remove the cover, and wash plate 3 times with 1X wash buffer.
9. Add 0.1 ml of SABC working solution into each well. Cover the plate and incubate at 37°C for 30 min.
10. Remove the cover and wash plate 5 times with 1X wash buffer. Each time let the wash buffer stay in the well for 1-2 min.
11. Add 90 µl of TMB substrate into each well, cover the plate and incubate at 37°C in dark within 15-30 min. (**Note:** This incubation time is for reference use only. The optimal time should be determined by end user). The shades of blue can be seen in the first 3-4 wells, only on most concentrated standards. Other wells show no obvious color.
12. Add 50 µl of stop solution into each well and mix thoroughly. Color will change into yellow immediately.
13. Read O.D. absorbance at 450 nm in a micro-plate reader immediately after adding the stop solution.

14. Calculation: Relative O.D. 450 = O.D. for each well – O.D. 450 control zero well. The Standard curve can be plotted as the relative O.D. 450 of each standard solution in Y axis vs. the respective concentration of the standard in X axis. Concentration of the samples can be incorporated from the standard curve. If the samples were diluted, multiply the dilution factor to the concentration.

Table-1

| | Standard 1 | Standard 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-------------|-------------|---|---|---|---|---|---|---|----|----|----|
| A | 5000 pg/ml | 5000 pg/ml | | | | | | | | | | |
| B | 2500 pg/ml | 2500 pg/ml | | | | | | | | | | |
| C | 1250 pg/ml | 1250 pg/ml | | | | | | | | | | |
| D | 625 pg/ml | 625 pg/ml | | | | | | | | | | |
| E | 312.5 pg/ml | 312.5 pg/ml | | | | | | | | | | |
| F | 156.25pg/ml | 156.25pg/ml | | | | | | | | | | |
| G | 78.12 pg/ml | 78.12 pg/ml | | | | | | | | | | |
| H | 0 | 0 | | | | | | | | | | |

IX. REFERENCES

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PMID: 19048177
2. Epigenetic silencing of TIMP4 in heart failure.
PMID: 27396717
3. Mice with tissue inhibitor of metalloproteinases 4 (Timp4) deletion succumb to induced myocardial infarction but not to cardiac pressure overload.
PMID: 20516072

4. TIMP4 Modulates ER- α Signaling in MCF7 Breast Cancer Cells.

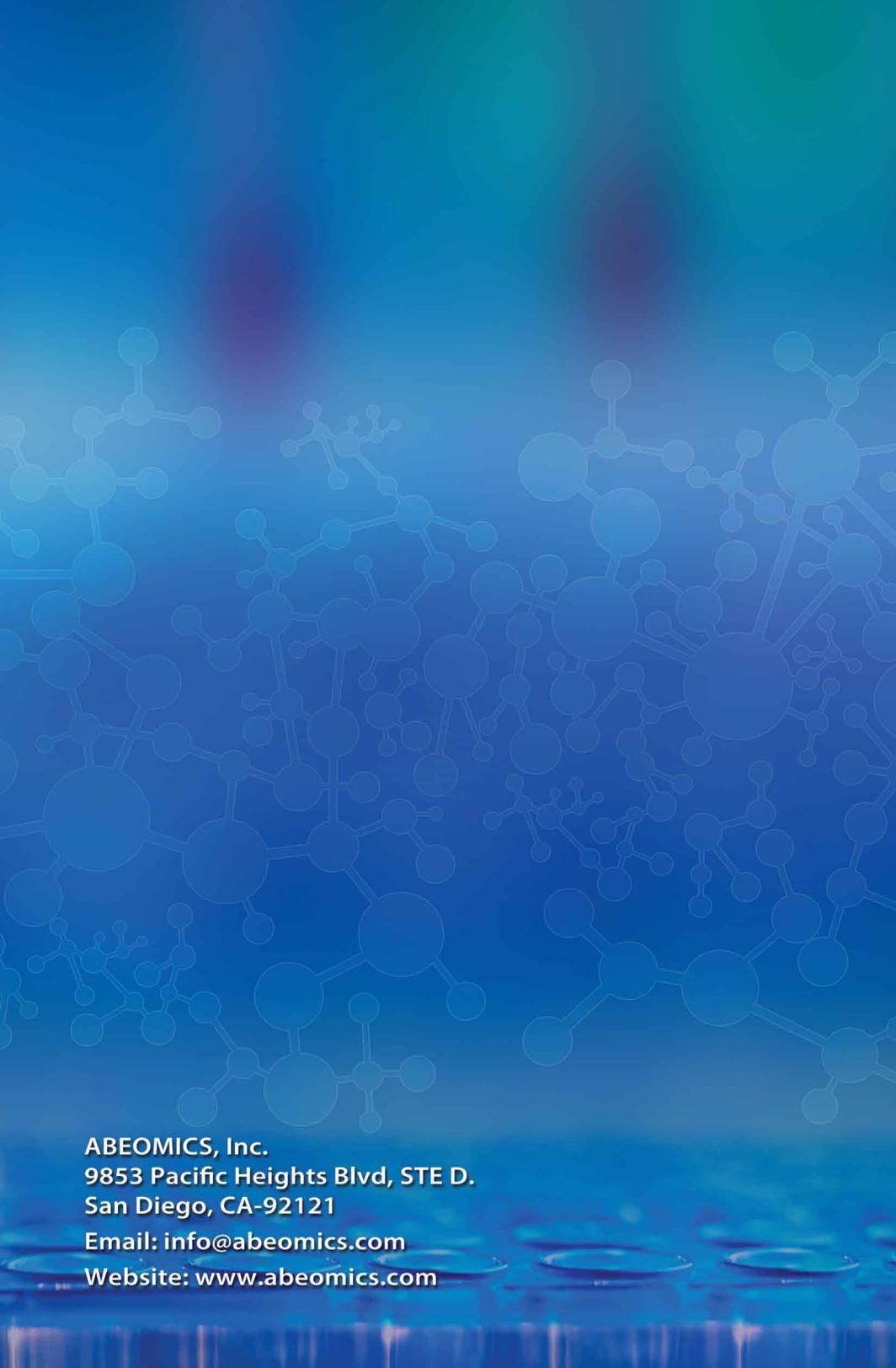
PMID: 27187039

5. Tissue inhibitor of matrix metalloproteinases 4 (TIMP4) in a population of young adults: relations to cardiovascular risk markers and carotid artery intima-media thickness. The Cardiovascular Risk in Young Finns Study.

PMID: 22978719

X. TROUBLE SHOOTING

| Problem | Probable Cause | Suggestion |
|-----------------|-------------------------------|---|
| No signal | Forgot to add all components. | Prepare check list and add the components in the correct order. |
| Low signal | Not enough lysates per well. | Check the protein concentration. Add more lysates. |
| High background | Washing is not sufficient. | Wash plates thoroughly after incubation with Streptavidin-HRP secondary |



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