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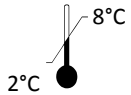
# IMUBIND® Thrombomodulin ELISA

**REF 837**


## INTENDED USE

The IMUBIND® Thrombomodulin ELISA is an enzyme linked immunosorbent assay for the measurement of thrombomodulin in human plasma, serum and cell culture supernatants. The ELISA measures whole and truncated forms of thrombomodulin as well as thrombomodulin/thrombin complexes, but is less sensitive to non-functional or degraded fragments.

This assay is for Research Use Only. It is not intended for either diagnostic or therapeutic use purposes in the United States of America.

## EXPLANATION OF THE TEST

Thrombomodulin (TM) is the cell surface receptor for thrombin. When occupied, thrombomodulin converts thrombin from a procoagulant protein into the activator of Protein C.[1,2] Once activated Protein C (APC) has been generated, thrombomodulin acts as a major anticoagulant through its ability to inactivate various blood factors (Va, VIIIa, Xa and XIIIa). In competing for thrombin binding, thrombomodulin inhibits the proteolytic effect of thrombin in its clotting of fibrinogen, the inactivation of Protein S and the induction of platelet aggregation.

TM is an integral membrane glycoprotein resembling in structure the low-density lipoprotein (LDL) receptor. TM possesses several EGF repeats, of which numbers five and six are responsible for the high affinity binding of thrombin ($K_d$ = 0.5 nM). In addition, the B chain of thrombin possesses a domain, distinct from the active catalytic site, termed anion-binding Exosite I, which is involved in the binding of thrombin to thrombomodulin. Also, TM contains a chondroitin sulfate (glycosoaminoglycan) which accelerates the inactivation of thrombin by anti-thrombin III. On SDS-polyacrylamide gels, human thrombomodulin appears as a single band at $M_r$ 75,000 D under non-reducing conditions and shows a band at approximately $M_r$ 110,000 D following reduction of its disulfide bonds.[3]

The thrombomodulin-thrombin complex enhances the catalytic activation of Protein C over 1,000 fold.[3] The binding of thrombin to thrombomodulin does not require calcium; however, interaction of the complex with Protein C is calcium dependent. Platelets, monocytes and neutrophils contain small amounts of TM in comparison to cultured endothelial cells. Immunohistochemical analysis has localized TM to the luminal surface of endothelium of blood vessels and lymphatics, the squamous epithelium, and the placental syncytiotrophoblast.[4]

TM is present in human plasma and urine in a truncated form, lacking the transmembrane and cytoplasmic domains of TM found on the cell surface. A detailed analysis of thrombomodulin circulating in human plasma revealed smaller fragments or degraded forms that are considered to possess only limited function. The concentration of these fragments was found to be increased in certain disease states.

Plasma levels of TM have been used as a marker for *in vivo* endothelial cell injury. In addition, TM may serve as a marker for transformal cells.[5] Cell surface expression of TM can be suppressed by treatment of endothelial cells with Tumor Necrosis Factor (TNF), interleukin I or endotoxin. These mediators induce the expression of tissue factor by endothelial cells, creating a procoagulant region on the endothelial cell surface, in opposition to the normally anticoagulant region.

## PRINCIPLE OF THE METHOD

The IMUBIND Thrombomodulin ELISA is a "sandwich" ELISA employing a monoclonal antibody which recognizes the $EGF_1$ - $EGF_2$ domains of TM. Specificity of the capture antibody for native, complexed and truncated TM was confirmed by Western Blot analysis. Samples incubate in microwells precoated with the capture antibody. A second horseradish peroxidase (HRP) conjugated monoclonal antibody specific for the $EGF_5$ – $EGF_6$ domains recognizes the bound TM, completing the antibody-antigen-antibody "sandwich".

The addition of a perborate/ 3,3',5,5' - tetramethylbenzidine (TMB) substrate and its subsequent reaction with the HRP creates a blue colored solution. Sensitivity is enhanced by addition of a sulfuric acid stop solution, turning the solution color yellow. TM levels are determined by measuring solution absorbances at 450 nm and comparing the values with those of a standard curve.

## REAGENTS

96 antibody coated microwells (6 x 16 well strips) and cover
2 vials of Thrombomodulin Depleted Plasma, 0.5 mL (lyophilized)
1 vial of Thrombomodulin Control, 1.0 mL (lyophilized)
2 vials of Thrombomodulin Standard, 10 ng/mL (lyophilized)
1 vial of HRP-Conjugated Detection Antibody (250 µL)
1 vial of Detection Antibody Diluent, 15 mL (lyophilized)
2 vials of Substrate, 11 mL
1 packet Wash Buffer, PBS Buffer with 0.05% Tween 20, pH 7.4, 1 Liter (powder)

## WARNINGS AND PRECAUTIONS

The Thrombomodulin Depleted Plasma included in this kit is of human origin. Each donor unit has been tested by an FDA approved method and found to be non-reactive for HBsAg, HIV-1 and HCV. As no known method can offer complete assurance that products derived from human blood will not transmit disease, this plasma should be handled as recommended for any potentially infectious human serum or blood specimen.

FOR RESEARCH USE ONLY. Do not use kit components beyond the printed expiry date. Do not mix reagents from different kit lots. Avoid microbial contamination of kit components. Do not mouth pipette or ingest any of the reagents. Do not smoke, eat or drink in areas where specimens or kit reagents are handled.

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|---|---|---|---|
| Thrombomodulin Standard & Thrombomodulin Control | Danger (US) Warning (EU) |  | H319, H334, H317, P264, P280, P261, P302 + P352, P333 + P313, P305 + P351 + P338, P337 + P313 |
| Detection Antibody | Warning |  | H319, P264, P280, P305 + P351 + P338, P337 + P313 |



| | | | |
|---|---|---|---|
| Detection Antibody Diluent | Danger |  | CONT: Polyethylene glycol octylphenol ether<br>H318, H334, H317, H412, P261, P273, P280, P302 + P352, P305 + P351 + P338, P333 + P313 |
| Wash buffer | Warning |  | H319, P264, P280, P305 + P351 + P338, P337 + P313 |

**Hazard Statements:**

H317 May cause an allergic skin reaction.
H318 Causes serious eye damage.
H319 Causes serious eye irritation.
H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled.
H412 Harmful to aquatic life with long lasting effects.

**Precautionary Statements:**

P261 Avoid breathing mist or vapor.
P264 Wash thoroughly after handling.
P273 Avoid release to the environment.
P280 Wear eye protection/face protection. Wear protective gloves.
P302 + P352 If on skin: Wash with plenty of water.
P305 + P351 + P338 If in eyes: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P333 + P313 If skin irritation or rash occurs: Get medical advice/ attention.
P337 + P313 If eye irritation persists: Get medical advice/attention.

## REAGENT PREPARATION AND STORAGE

**Procedural Notes:**

1. Accurate measurement of pipetting volumes is critical for valid results.
2. Reconstitute Thrombomodulin Standard and Thrombomodulin Control immediately before adding to the microwells. DO NOT prepare standards or control in advance and store at 2° – 8°C.
3. Aliquot remaining Thrombomodulin Standard, Thrombomodulin Depleted Plasma and Thrombomodulin Control and store at -20°C.

### A. Thrombomodulin Standard

1. Add 0.5 mL of cold (2°– 8°C) filtered deionized or distilled water to each of the Thrombomodulin Depleted Plasma vials. Allow the vial to stand on ice for 2–3 minutes. Vortex the vial to achieve adequate mixing.

2. Prepare a solution of 5% thrombomodulin depleted plasma solution by adding the Thrombomodulin Depleted Plasma (2 vials, 1 mL) to 19 mL of cold filtered deionized or distilled water. Gently vortex and let stand for 5 minutes.
3. Hold the 10 ng/mL Thrombomodulin Standard vial upright and tap the vial to settle its contents (lyophilized under vacuum). Release the vacuum by slowly removing the vial stopper.
4. Add 1 mL of the 5% thrombomodulin depleted plasma to the 10 ng/mL standard vial.
5. Serially dilute the 10 ng/mL thrombomodulin standard to prepare standards with concentrations of 5, 2.5, 1.25 and 0.625 ng/mL (label tubes as such). Pipette 0.5 mL of 5% thrombomodulin depleted plasma into each tube. Pipette 0.5 mL of the 10 ng/mL thrombomodulin standard into the 5 ng/mL labeled tube and mix. Transfer 0.5 mL from the 5 ng/mL tube into the 2.5 ng/mL labeled tube and mix. Continue this process for the 1.25 ng/mL and 0.625 ng/mL labeled tubes.
6. Use the 5% thrombomodulin depleted plasma as the 0 ng/mL standard.

**B. Thrombomodulin Control**

Add 1.0 mL of filtered deionized or distilled water to the vial and gently mix for 2 minutes.

**C. Detection Antibody Diluent**

1. Add 15 mL of filtered deionized or distilled water to the Detection Antibody Diluent vial and mix well.
2. Add the 15 mL of diluent to another 15 mL of filtered deionized or distilled water and mix well.

**D. Detection Antibody**

Add 10 µL of HRP conjugated Detection Antibody per mL of Detection Antibody Diluent needed (200 µL per microwell).

**E. Wash Buffer**

1. Dissolve the contents of the Wash Buffer packet in 900 mL of filtered deionized or distilled water and mix well.
2. Dilute to a final volume of 1 Liter with filtered deionized or distilled water.

**F. Sample Buffer**

Prepare an appropriate amount of Sample Buffer by adding BSA to Wash Buffer to a final concentration of 1% w/v (1 gm BSA/100 mL Wash Buffer).



## REAGENT STABILITY

Store unused microwells and lyophilized reagents at 2° – 8°C until expiration dates indicated on label.

Store reconstituted reagents at -20°C for up to one month.

## SPECIMEN COLLECTION AND PREPARATION

**A. Plasma**

Use citrate collected platelet poor plasma for this assay. See "Collection, Transport and Processing of Blood Specimens for Testing Plasma-based Coagulation Assays and Molecular Hemostasis Assays; Approved Guidelines – Fifth Edition", CLSI document H21-A5, Vol. 28, No. 5, January 2008. Plasma collection should be performed as follows:

1. Collect 9 parts of blood into 1 part of 3.2% (0.109 M) trisodium citrate anticoagulant solution.
2. Centrifuge the blood sample at 3,000 x g for 15 minutes.
3. Plasma should be stored at room temperature and assayed within 2 hours. Alternatively, plasma may be stored below -70°C for up to 6 months
4. Frozen plasma should be thawed rapidly at 37°C. Thawed plasma should be stored at room temperature and assayed within 2 hours.
5. Dilute plasma sample and Thrombomodulin Control 1:4 in Sample Buffer.

**B. Tissue Culture Supernatant**

Dilute sample 1:5 (recommended initial dilution) in Sample Buffer. Note: some cell systems may require a higher dilution factor.

## PROCEDURE

**Materials Provided** – See Reagents

**Materials Required But Not Provided**

filtered deionized or distilled water
50-200 µL eight channel multi-pipette, 10-200 µL single pipette
Microwell plate reader at 450 nm
Microwell plate washer
0.5M $H_2SO_4$
Bovine Serum Albumin (BSA)

**Assay Procedure**

1. Remove the necessary number of precoated microwells from the foil pouch and place them in the plate holder. Replace and seal unused microwells in the foil pouch with the desiccant inside and store at 2°- 8°C.

2. Add 200 µL of standard, diluted control or diluted plasma sample to the microwells cover with the lid and incubate for 1 hour at room temperature. Perform measurement in duplicate.
3. Wash wells 4 times with Wash Buffer.
4. Add 200 µL of Detection Antibody to each well, cover with lid and incubate for 30 minutes at room temperature.
5. Wash wells 4 times with Wash Buffer.
6. Add 200 µL of Substrate solution to each well, cover with lid and incubate for 20 minutes at room temperature. A blue color will develop.
7. Stop the enzymatic reaction by adding 100 µL of 0.5M $H_2SO_4$ to each well. The solution color will turn yellow. Read the absorbances on a micro-test plate reader at a wavelength of 450 nm within 30 minutes. Deduct the background average of the blanks from the standards and sample readings.

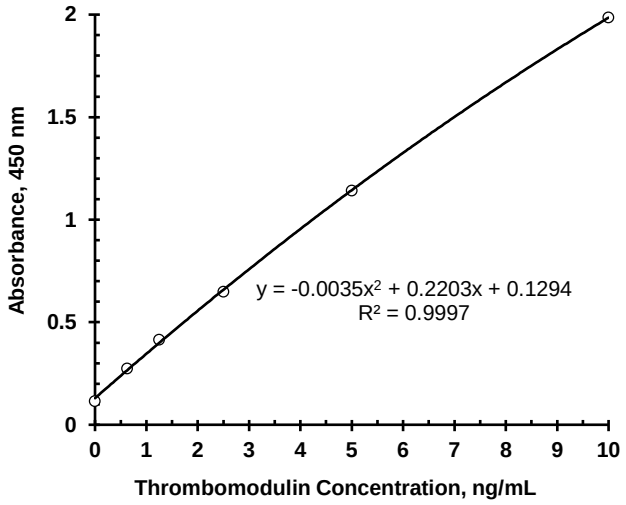
## RESULTS

### Representative Standard Curve

The standard curve is constructed by plotting the mean absorbance value for each thrombomodulin standard versus the corresponding concentration of thrombomodulin in ng/mL. A standard curve should be generated each time the assay is performed and a 2nd order polynomial equation should be used for the regression analysis. Interpolate the concentrations for the diluted samples directly from the standard curve.

The following standard curve is for demonstration purposes only.

**IMUBIND® Thrombomodulin ELISA**






## CALCULATION OF RESULTS

Average thrombomodulin concentrations obtained for each test sample, as interpolated from the standard curve. Multiply this concentration by the dilution factor of the sample to calculate thrombomodulin concentration of original sample. For example, if the test sample was diluted 1:4 as recommend for plasma, multiply the concentration of the diluted sample read from standard curve by 4 to obtain the actual concentration. The calculation would be:

Concentration of Test Sample = Concentration of Diluted Test Sample x 4

## REFERENCES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7. Takano, S., Kimura, S., Ohdama, S., and Aoki, N. Plasma Thrombomodulin in Health and Diseases. *Blood* 1990; **76**: 2024-2029.


## DEFINITION OF SYMBOLS

| | | | |
|---|---|---|---|
| | Consult instructions for use | REF | Catalog Number |
| | Manufacturer | LOT | Lot Number |
| | Refer to Safety Data Sheet | | Expiration Date |
| 2°C 8°C | Store at 2°C to 8°C | Σ n | Contains sufficient for <n> tests |
| CONT | contains | | |