

american diagnostica inc.

500 West Avenue, P.O. Box 110215 Stamford, CT. 06911-0215
Tel. (203) 602-7777 • Fax. (203) 602-2221
www.americandiagnostica.com

IMUBIND[®] Thrombomodulin ELISA Kit

Product No. 837

INTENDED USE

The IMUBIND[®] Thrombomodulin ELISA Kit is an enzyme linked immunosorbent assay for the quantitation 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 clinical diagnostic or therapeutic use purposes in the United States of America.

Background

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 (glycosaminoglycan) 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 bonds³.

The thrombomodulin-thrombin complex enhances the catalytic activation of Protein C over 1,000 fold³. 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 syncytiotrophoblast⁴.

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 plasma⁷ 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 cells⁵. 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

The IMUBIND Thrombomodulin ELISA is a "sandwich" ELISA employing a monoclonal antibody which recognizes the EGF₁ - EGF₂ domains of TM. Specificity of the capture antibody for native, complexed and truncated TM was confirmed by Western Blot analysis. Samples incubate in microtest wells precoated with the capture antibody. A second horseradish peroxidase (HRP) conjugated monoclonal antibody specific for the EGF₅ - EGF₆ 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.

Kit Reagents

6 x 16 well precoated microtest strips with holder and lid
2 vials Thrombomodulin Depleted Plasma (lyophilized)
1 vial Thrombomodulin Reference Plasma
2 vials Thrombomodulin Standard, 10 ng/mL (lyophilized)
1 vial HRP-Conjugated Detection Antibody (250 µL)
1 vial Detection Antibody Diluent (lyophilized)
1 vial Substrate (22 mL)
1 packet PBS Buffer, 0.05% Tween 20, pH 7.4

Additional Materials Required but not Provided

Distilled H₂O
50 - 200 µL eight channel multi-pipette
10 - 200 µL single pipette
Micro-test plate reader at 450 nm
0.5M H₂SO₄
Bovine Serum Albumin (BSA, e.g. Sigma A-7030)

WARNING

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.

Reagent Preparation

Procedural Notes:

1. Accurate measurement of pipetting volumes is critical for valid results.
2. Reconstitute standards and references immediately before adding to the microtest wells. **DO NOT prepare standards in advance and store at 4°C.**
3. Aliquot remaining standards, TM depleted plasma and TM reference plasma and store at -20°C.

A. Standards

1. Add 0.5 mL of cold (+2 - +8°C) distilled H₂O to each of the TM 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% TM depleted plasma solution by adding the TM depleted plasmas (2 vials, 1 mL) to 19 mL cold H₂O. Gently vortex and let stand for 5 minutes.
3. Hold the 10 ng/mL TM 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% TM depleted plasma to the 10 ng/mL standard vial.
5. Serially dilute the 10 ng/mL TM plasma standard to generate standards with concentrations of 5, 2.5, 1.25 and 0.625 ng/mL (label test tubes as such). Pipette 0.5 mL of 5% TM depleted plasma into each tube. Pipette 0.5 mL of the 10 ng/mL TM plasma 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% TM depleted plasma as the "0" standard.

B. TM Reference Plasma

Add 0.5 mL of cold distilled H₂O to the vial and gently mix for 2 minutes.

C. Detection Antibody Diluent

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

E. Wash Buffer

1. Dissolve contents of PBS/0.05% Tween 20 packet in 900 mL of distilled H_2O .
2. Mix well.
3. Dilute to a final volume of 1 Liter with distilled H_2O .

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 micro-test strip-wells and unreconstituted reagents at $+2^\circ - +8^\circ\text{C}$ until expiration dates indicated on label.

Store reconstituted reagents at -20°C for up to one month. Remember to aliquot and freeze reconstituted standards and plasmas **immediately**.

Sample Preparation

A. Plasma (Note: Heparinized plasma can be used in this assay)

1. Collect blood into 3.8% trisodium citrate anticoagulant solution in the proportion of 9 volumes of blood to 1 volume of anticoagulant solution.
2. Centrifuge the blood sample at 3,000 rpm for 10 minutes and store frozen.
3. Frozen plasma should be thawed at 37°C .
4. Dilute TM Reference Plasma and plasma samples 1:4 in Sample Buffer.

B. Tissue culture supernatants

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

ASSAY PROCEDURE

1. Remove the necessary number of precoated micro-test strips from the foil pouch and place them in the plate holder. Reseal unused strips in the foil pouch with the desiccant inside and store at $+2^\circ - +8^\circ\text{C}$.
2. Add 200 μL of **Thrombomodulin Standard**, diluted **Reference Plasma** or diluted plasma sample to the micro-test wells, 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 . 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.

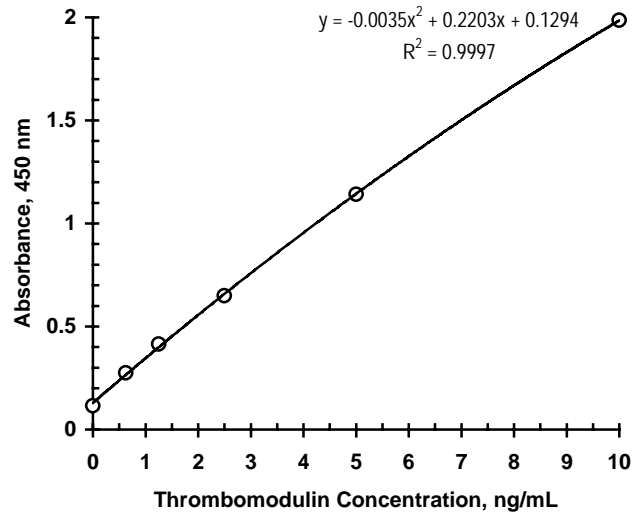
INTERPRETATION OF RESULTS

A normal range for human plasma has yet to be established. Citrated plasma samples from healthy volunteers ($n=50$) were found to have varying thrombomodulin levels depending upon sex and age. Thrombomodulin levels in males ranged from mean values of 4.00-5.35 ng/mL, independent of age. Mean levels in females ranged from 2.73 ng/mL for the age group 21-30 years increasing to 4.79 ng/mL for the age group 61-70 years.⁶

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. Interpolate the concentrations for the diluted samples directly from the standard curve. A standard curve should be generated each time the assay is performed. 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

Performance Characteristics

Specificity: This assay recognizes native and truncated functional forms of thrombomodulin and thrombomodulin/thrombin complexes. Preparations of various coagulation factors (at 10-fold concentrations of TM) in both 5% TM Depleted Plasma and Sample Buffer were assayed. No significant cross-reactivity or interference was observed.

Sensitivity: The lower limit of detection was determined by adding 2 standard deviations to the mean OD value for the "0" standard (n=20) and calculating the corresponding concentration from the standard curve. For plasma samples, the lower limit of detection was found to be 0.300 ng Thrombomodulin/mL.

Precision: Intra-assay Coefficient of Variation = 4.0% (n=20)
Inter-assay Coefficient of Variation = 5.2% (n=20)

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