

Human Thrombomodulin ELISA Kit

Catalog No. EA100576
 Size 96T(8x12 divisible strips)

For quantitative detection of human Thrombomodulin in cell culture supernates, serum, plasma(heparin, EDTA, citrate) and urine.

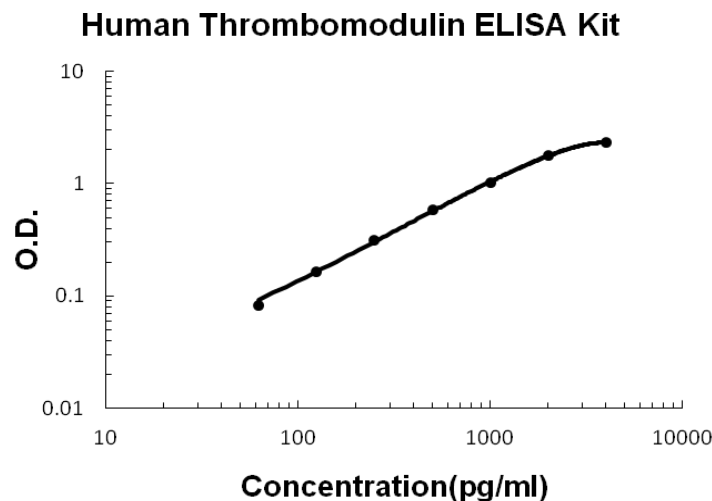
Typical Data Obtained from Human Thrombomodulin

(TMB reaction incubate at 37°C for 20 min)

Concentration(pg/ml)	0.0	62.5	125	250	500	1000	2000	4000
O.D	0.008	0.083	0.165	0.311	0.584	1.024	1.774	2.317

Typical Human Thrombomodulin ELISA Kit Standard Curve

This standard curve was generated at OriGene for demonstration purpose only. A standard curve must be run with each assay.



Range	62.5pg/ml-4000pg/ml
Sensitivity	< 10pg/ml
Specificity	Natural and recombinant human Thrombomodulin
Cross-reactivity	No detectable cross-reactivity with other relevant proteins

Storage

Store at 4°C for 6 months, at -20°C for 12 months. Avoid multiple freeze-thaw cycles (Shipped with wet ice.)

Precision

Intra-Assay Precision (Precision within an assay) Three samples of known concentration were tested on one plate to assess intra-assay precision.

Inter-Assay Precision (Precision between assays) Three samples of known concentration were tested in separate assays to assess inter-assay precision.

Sample	Intra-Assay Precision			Inter-Assay Precision		
	1	2	3	1	2	3
n	16	16	16	24	24	24
Mean(pg/ml)	523	1074	2246	576	1187	2315
Standard deviation	24.1	40.1	92.1	47.2	86.7	180.6
CV(%)	4.6	3.7	4.1	8.2	7.3	7.8

Principle

OriGene's human Thrombomodulin ELISA Kit was based on standard sandwich enzyme-linked immune-sorbent assay technology. A monoclonal antibody from mouse specific for Thrombomodulin has been precoated onto 96-well plates. Standards(NSO, A19-S515) and test samples are added to the wells, a biotinylated detection polyclonal antibody from goat specific for Thrombomodulin is added subsequently and then followed by washing with PBS or TBS buffer. Avidin-Biotin-Peroxidase Complex was added and unbound conjugates were washed away with PBS or TBS buffer. HRP substrate TMB was used to visualize HRP enzymatic reaction. TMB was catalyzed by HRP to produce a blue color product that changed into yellow after adding acidic stop solution. The density of yellow is proportional to the human Thrombomodulin amount of sample captured in plate.

Kit Components

Description	Quantity
96-well plate precoated with anti- human Thrombomodulin antibody	1
Lyophilized recombinant human Thrombomodulin standard	10ng/tubex2
Biotinylated anti- human Thrombomodulin antibody	130µl(dilution 1:100)
Avidin-Biotin-Peroxidase Complex (ABC)	130µl(dilution 1:100)
Sample diluent buffer	30 ml
Antibody diluent buffer	12ml
ABC diluent buffer	12ml
TMB color developing agent	10ml
TMB stop solution	10ml

Material Required But Not Provided

1. Microplate reader in standard size.
2. Automated plate washer.
3. Adjustable pipettes and pipette tips. Multichannel pipettes are recommended in the condition of large amount of samples in the detection.
4. Clean tubes and Eppendorf tubes.
5. Washing buffer (neutral PBS or TBS).
 - Preparation of 0.01M **TBS**: Add 1.2g Tris, 8.5g NaCl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 1000ml H₂O and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.
 - Preparation of 0.01 M **PBS**: Add 8.5g sodium chloride, 1.4g Na₂HPO₄ and 0.2g NaH₂PO₄ to 1000ml distilled water and adjust pH to 7.2-7.6. Finally, adjust the total volume to 1L.

Notice for Application of Kit

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. The TMB Color Developing agent is colorless and transparent before using, contact us freely if it is not the case.
3. Before using the Kit, spin tubes and bring down all components to the bottom of tubes.
4. Duplicate well assay is recommended for both standard and sample testing.
5. Don't let 96-well plate dry, for dry plate will inactivate active components on plate.
6. Don't reuse tips and tubes to avoid cross contamination.
7. Avoid using the reagents from different batches together.
8. In order to avoid marginal effect of plate incubation due to temperature difference (reaction may be stronger in the marginal wells), it is suggested that the diluted ABC and TMB solution will be pre-warmed in 37°C for 30 min before using.

Preparation

1. Sample Preparation and Storage

Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C.

Avoid repeated freeze-thaw cycles.

- **Cell culture supernates:** Remove particulates by centrifugation, assay immediately or aliquot and store samples at -20°C.
- **Serum:** Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1000 X g for 15 min. Analyze the serum immediately or aliquot and store frozen at -20°C.
- **Plasma:** Collect plasma using **heparin, EDTA or Citrate** as an anticoagulant. Centrifuge for 15 min at 1000 x g within 30 min of collection. Analyze immediately or aliquot and store frozen at -20°C. Citrate is not recommended as the anticoagulant.
- **Urine:** Aseptically collect the first urine of the day, micturate directly into a sterile container. Remove particular impurities by centrifugation, assay immediately or aliquot and store samples at -20°C.

2. Sample Dilution Guideline

The user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of the linear regime in the standard curve. Dilute the sample using the provided diluent buffer. The following is a guideline for sample dilution. Several trials may be necessary in practice. **The sample must be well mixed with the diluents buffer.**

- **High target protein concentration (40-400ng/ml).** The working dilution is 1:100. i.e. Add 3 µl sample into 297 µl sample diluent buffer.
- **Medium target protein concentration (4-40ng/ml).** The working dilution is 1:10. i.e. Add 25 µl sample into 225 µl sample diluent buffer.
- **Low target protein concentration (62.5-4000pg/ml).** The working dilution is 1:2. i.e. Add 100 µl sample to 100 µl sample diluent buffer.
- **Very Low target protein concentration (≤62.5pg/ml).** No dilution necessary, or the working dilution is 1:2.

3. Reagent Preparation and Storage

- A. Reconstitution of the human Thrombomodulin standard: Thrombomodulin standard solution should be prepared no more than 2 hours prior to the experiment. Two tubes of Thrombomodulin standard (10ng per

tube) are included in each kit. Use one tube for each experiment.

- a. 10,000pg/ml of human Thrombomodulin standard solution: Add 1 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
- b. 4000pg/ml of human Thrombomodulin standard solution: Add 0.4ml of the above 10ng/ml Thrombomodulin standard solution into 0.6ml sample diluent buffer and mix thoroughly.
- c. 2000pg/ml→62.5pg/ml of human Thrombomodulin standard solutions: Label 6 Eppendorf tubes with 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml respectively. Aliquot 0.3ml of the sample diluent buffer into each tube. Add 0.3ml of the above 4000pg/ml Thrombomodulin standard solution into 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3ml from 2nd tube to 3rd tube and mix, and so on.

Note: The standard solutions are best used within 2 hours. The 10ng/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

- B. Preparation of biotinylated anti-human Thrombomodulin antibody working solution: The solution should be prepared no more than 2 hours prior to the experiment.
 - a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
 - b. Biotinylated anti-human Thrombomodulin antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly. (i.e. Add 1µl Biotinylated anti-human Thrombomodulin antibody to 99µl antibody diluent buffer.)
- C. Preparation of Avidin-Biotin-Peroxidase Complex (ABC) working solution: The solution should be prepared no more than 1 hour prior to the experiment.
 - a. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
 - b. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly. (i.e. Add 1µl ABC to 99µl ABC diluent buffer.)

Assay Procedure

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard Thrombomodulin detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of Thrombomodulin amount in samples.

1. Aliquot 0.1ml per well of the 4000pg/ml, 2000pg/ml, 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml human Thrombomodulin standard solutions into the precoated 96-well plate. Add 0.1ml of the sample diluent buffer into the control well (Zero well). Add 0.1ml of each properly diluted sample of human cell culture supernates, serum, plasma(heparin, EDTA, citrate) or urine to each empty well. **See “Sample Dilution Guideline” above for details.** We recommend that each human Thrombomodulin standard solution and each sample is measured in duplicate.
2. Seal the plate with the cover and incubate at 37°C for 90 min.
3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 0.1ml of biotinylated anti-human Thrombomodulin antibody working solution into each well and incubate the plate at 37°C for 60 min.
5. Wash plate 3 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. **(Plate Washing Method:** Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1~2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For

- automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.)
6. Add 0.1ml of prepared ABC working solution into each well and incubate the plate at 37°C for 30 min.
 7. Wash plate 5 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (See Step 5 for plate washing method).
 8. Add 90µl of prepared TMB color developing agent into each well and incubate plate at 37°C in dark for 20-25 min (**Note:** For reference only, the optimal incubation time should be determined by end user. And the shades of blue can be seen in the wells with the four most concentrated human Thrombomodulin standard solutions; the other wells show no obvious color).
 9. Add 0.1ml of prepared TMB stop solution into each well. The color changes into yellow immediately.
 10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution.

For calculation, (the relative O.D.₄₅₀) = (the O.D.₄₅₀ of each well) – (the O.D.₄₅₀ of Zero well). The standard curve can be plotted as the relative O.D.₄₅₀ of each standard solution (Y) vs. the respective concentration of the standard solution (X). The human Thrombomodulin concentration of the samples can be interpolated from the standard curve. **Note:** if the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

Summary

1. Add samples and standards and incubate the plate at 37°C for 90 min. Do not wash.
2. Add biotinylated antibodies and incubate the plate at 37°C for 60 min. Wash plate 3 times with 0.01M TBS.
3. Add ABC working solution and incubate the plate at 37°C for 30 min. Wash plate 5 times with 0.01M TBS.
4. Add TMB color developing agent and incubate the plate at 37°C in dark for 20-25 min.
5. Add TMB stop solution and read.

Background

Thrombomodulin (TM), CD141 or BDCA-3 is an integral membrane protein expressed on the surface of endothelial cells and serves as a cofactor for thrombin. It reduces blood coagulation by converting thrombin to an anticoagulant enzyme from a procoagulant enzyme. Thrombomodulin is also expressed on human mesothelial cell, monocyte and a dendritic cell subset. In humans, thrombomodulin is encoded by the THBD gene. The protein has a molecular mass of 74kDa, and consists of a single chain with 5 distinct domains. Thrombomodulin functions as a cofactor in the thrombin-induced activation of protein C in the anticoagulant pathway by forming a 1:1 stoichiometric complex with thrombin. This raises the speed of protein C activation thousandfold. Thrombomodulin-bound thrombin has procoagulant effect at the same time by inhibiting fibrinolysis by cleaving thrombin-activatable fibrinolysis inhibitor (TAFI, aka carboxypeptidase B2) into its active form. Thrombomodulin is a glycoprotein on the surface of endothelial cells that, in addition to binding thrombin, regulates C3b inactivation by factor I. Mutations in the thrombomodulin gene (THBD) have also been reported to be associated with atypical hemolytic-uremic syndrome (aHUS).

Reference

1. IPR001491 Thrombomodulin Accessed January 19, 2012.
2. <http://www.ncbi.nlm.nih.gov/pubmed/8943899>.
3. Wen DZ, Dittman WA, Ye RD, Deaven LL, Majerus PW, Sadler JE (July 1987). "Human thrombomodulin: complete cDNA sequence and chromosome localization of the gene". *Biochemistry* 26 (14): 4350–7.