OriGene Technologies Inc.
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Human Thrombomodulin ELISA Kit

Catalog Number: EA100576

Assay Principle

The OriGene Human THBD Pre-Coated ELISA (Enzyme-Linked Immunosorbent Assay) kit is a solid-phase immunoassay specially designed to measure Human THBD with a 96-well strip plate that is pre-coated with antibody specific for THBD. The detection antibody is a biotinylated antibody specific for THBD. The capture antibody is monoclonal antibody from mouse and the detection antibody is polyclonal antibody from goat.The kit includes Human THBD protein as standards.

To measure Human THBD, add standards and samples to the wells, then add the biotinylated detection antibody. Wash the wells with PBS or TBS buffer, and add Avidin-Biotin-Peroxidase Complex (ABC-HRP). Wash away the unbounded ABC-HRP with PBS or TBS buffer and add TMB. TMB is an HRP substrate and will be catalyzed to produce a blue color product, which changes into yellow after adding the acidic stop solution. The absorbance of the yellow product at 450nm is linearly proportional to Human THBD in the sample. Read the absorbance of the yellow product in each well using a plate reader, and benchmark the sample wells' readings against the standard curve to determine the concentration of Human THBD in the sample.

Overview

Product Name	Human Thrombomodulin ELISA
Reactive Species	Human
Size	96wells/kit, with removable strips.
Description	Sandwich High Sensitivity ELISA kit for Quantitate Human THBD in cell culture supernatants, serum and plasma (heparin, EDTA, citrate). Sensitivity: 10pg/ml.
Sensitivity	<10 pg/ml *The sensitivity or the minimum detectable dose (MDD) is the lower limit of target protein that can be detected by the kit. It is determined by adding two standard deviations to the mean O.D. value of twenty (20) blank wells and calculating the corresponding concentration.
Detection Range	62.5 pg/ml – 4000 pg/ml
Storage Instructions	Store at 4°C for 6 months, at -20°C for 12 months. Avoid multiple freeze-thaw cycles (Shipped with wet ice.)
Uniprot ID	P07204

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Technical Details

Capture/Detection Antibodies	*The capture antibody is monoclonal antibody from mouse and the detection antibody is polyclonal antibody from goat.*
Specificity	*Natural and recombinant Human THBD*
Immunogen	*Expression system for standard: E.coli; Immunogen sequence: A19-S515*
Cross Reactivity	*This kit is for the detection of Human THBD. No significant cross-reactivity or interference between THBD and its analogs was observed. This claim is limited by existing techniques; therefore, crossreactivity may exist with untested analogs.*

Notice Before Application

Please read the following instructions before starting the experiment.

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. *Before using the Kit, spin tubes and bring down all components to the bottom of tubes.*
3. *Don't let 96-well plate dry, for dry plate will inactivate active components on plate.*
4. *Don't reuse tips and tubes to avoid cross contamination.*
5. *Avoid using the reagents from different batches together.*

Kit Components/Materials Provided

Description	Quantity	Volume	Storage of open/reconstituted material
Anti-Human THBD Pre-coated 96-well strip microplate	***1***	***12 strips of 8 wells***	**Return unused wells to the foil pouch. Reseal along the entire edge of the zip-seal. May be stored for up to 1 month at 4°C provided this is within the expiration date of the kit.**
Human THBD Standard	***2***	***10 ng/tube***	**Discard the *THBD* stock solution after 12 hours at 4°C. May be stored at -20°C for 48 hours.**
Human THBD Biotinylated antibody (100x)	***1***	***130 µl***	**May be stored for up to 1 month at 4°C provided this is within the expiration date of the kit.**
Avidin-Biotin-Peroxidase Complex (100x)	***1***	***130 µl***	
Sample Diluent	***1***	***30ml***	
Antibody Diluent	***1***	***12ml***	
Avidin-Biotin-Peroxidase Diluent	***1***	***12ml***	
Color Developing Reagent (TMB)	***1***	***10ml***	
Stop Solution	***1***	***10ml***	
Wash Buffer(25x)	***1***	***20 ml***	
Plate Sealers	***4***	***Pieces***	

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Required Materials That Are Not Supplied

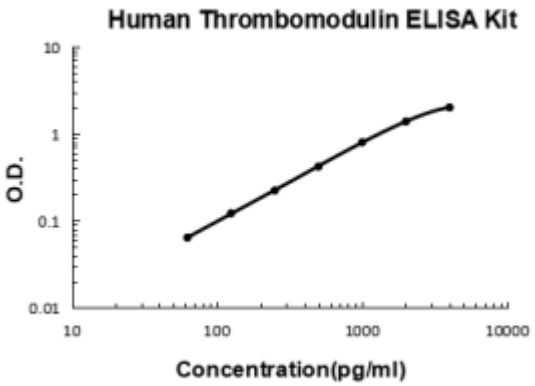
Microplate Reader capable of reading absorbance at 450nm.
Automated plate washer (optional)
Pipettes and pipette tips capable of precisely dispensing 0.5 µl through 1 ml volumes of aqueous solutions. Multichannel pipettes are recommended for large amount of samples.
Deionized or distilled water.
500ml graduated cylinders.
Test tubes for dilution.

Human THBD ELISA Kit Standard Curve Example

Highest O.D. value might be higher or lower than in the example. The experiment result is statistically significant if the highest O.D. value is no less than 1.0.

Concentration (pg/ml)	**0**	**62.5**	**125**	**250**	**500**	**1000**	**2000**	**4000**
O.D.	**0.001**	**0.065**	**0.123**	**0.224**	**0.421**	**0.801**	**1.387**	**2.010**

Human Thrombomodulin ELISA Kit standard curve



A standard curve is provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Intra/Inter Assay Variability

OriGene spend great efforts in documenting lot to lot variability and make sure our assay kits produce robust data that are reproducible.

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 across assays): *Three samples of known concentration were tested in separate assays to assess inter-assay precision.*

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	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	16	16	16	24	24	24
Mean(pg/ml)	164	418	1319	176	453	1243
Standard deviation	7.38	26.33	79.14	9.68	33.52	91.98
CV(%)	4.5	6.3	6	5.5	7.4	7.4

Reproducibility

To assay reproducibility, three samples with differing target protein concentrations were assayed using four different lots.

Lots	Lot1 (pg/ml)	Lot2 (pg/ml)	Lot3 (pg/ml)	Lot4 (pg/ml)	Mean (pg/ml)	Standard Deviation	CV (%)
Sample 1	164	165	162	171	165	3.35	2
Sample 2	418	416	394	403	407	9.8	2.4
Sample 3	1319	1304	1439	1459	1380	69.31	5

*number of samples for each test n=16.

Preparation Before The Experiment

Item	Preparation
All reagents	Bring all reagents to 37°C prior to use. The assay can also be done at room temperature however we recommend doing it at 37°C for best consistency with our QC results. Also the TMB incubation time estimate (15-25min) is based on 37°C. Do not equilibrate unused plate well strips to room temperature; these should be sealed and stored in the original packaging.
Wash buffer	Prepare 500 ml of working Wash Buffer by diluting the supplied 20 ml of Wash Buffer (25x) with 480 ml of deionized or distilled water. If crystals have formed in the concentrate, warm to room temperature and mix it gently until crystals have completely dissolved.
Biotinylated Anti-Human THBD antibody	It is recommended to prepare this reagent immediately prior to use by diluting the Human THBD Biotinylated antibody (100x) 1:100 with Antibody Diluent. Prepare 100 µl by adding 1 µl of Biotinylated antibody (100x) to 99 µl of Antibody Diluent for each well. Mix gently and thoroughly and use within 2 hours of generation.
Avidin-Biotin-Peroxidase Complex	It is recommended to prepare this reagent immediately prior to use by diluting the Avidin-Biotin-Peroxidase Complex (100x) 1:100 with Avidin-Biotin-Peroxidase Diluent. Prepare 100 µl by adding 1 µl of Avidin-Biotin-Peroxidase Complex (100x) to 99 µl of Avidin-Biotin-Peroxidase Diluent for each well. Mix gently and thoroughly and use within 2 hours of generation.
Human THBD Standard	It is recommended that the standards be prepared no more than 2 hours prior to performing the

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	experiment. Use one 10 ng of lyophilized Human THBD standard for each experiment. Gently spin the vial prior to use. Reconstitute the standard to a stock concentration of 10 ng/ml using 1ml of sample diluent. Allow the standard to sit for a minimum of 10 minutes with gentle agitation prior to making dilutions.
Microplate	*The included microplate is coated with capture antibodies and ready-to-use. It does not require additional washing or blocking. The unused well strips should be sealed and stored in the original packaging.*

Dilution of Human THBD Standard

1. *1. Number tubes 1-8. Final Concentrations to be Tube # 1: 4,000.00 pg/ml, # 2: 2,000.00 pg/ml, # 3: 1,000.00 pg/ml, # 4: 500.00 pg/ml, # 5: 250.00 pg/ml, # 6: 125.00 pg/ml, # 7: 62.50 pg/ml, # 8: Sample Diluent serves as the zero standard (0 pg/ml).*
2. *To generate standard #1, add 400 µl of the reconstituted standard stock solution of 10 ng/ml and 600 µl of sample diluent to tube #1 for a final volume of 1000 µl. Mix thoroughly.*
3. *Add 300 µl of sample diluent to tubes # 2-7.*
4. *To generate standard # 2, add 300 µl of standard # 1 from tube # 1 to tube # 2 for a final volume of 600 µl. Mix thoroughly.*
5. *To generate standard # 3, add 300 µl of standard # 2 from tube # 2 to tube # 3 for a final volume of 600 µl. Mix thoroughly.*
6. *Continue the serial dilution for tube # 4-7.*

Sample Preparation and Storage

These sample collection instructions and storage conditions are intended as a general guideline and the sample stability has not been evaluated.

Sample Type	*Procedure*
Cell culture supernatants	*Clear sample of particulates by centrifugation, assay immediately or store samples at -20°C.*
Serum	*Use a serum separator tube (SST) and allow serum to clot at room temperature for about four hours. Then, centrifuge for 15 min at approximately 1,000 x g. assay immediately or store samples at -20°C.*
Plasma	*Collect plasma using heparin, EDTA or Citrate as an anticoagulant. Centrifuge for 15 min at approximately 1,000 x g. Assay immediately or store samples at -20°C.* **Note: it is important to not use anticoagulants other than the ones described above to treat plasma, for other anticoagulants could block the antibody binding site.*
Urine	*Collect the first urine of the day, micturate directly into a sterile container. Remove impurities by centrifugation, assay immediately or aliquot and store samples at -20°C.*

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Sample Dilution

The user needs to estimate the concentration of the target protein in the sample and use an appropriate dilution factor so that the diluted target protein concentration falls in the range of O.D. values of the standard curve. Dilute the sample using provided diluent buffer. Pilot tests using a dilution series of each sample type are necessary. The sample must be mixed thoroughly with Sample Diluent.

Assay protocol

It is recommended that all reagents and materials be equilibrated to 37°C/room temperature prior to the experiment (see Preparation Before The Experiment if you have missed this information).

1. *Prepare all reagents and working standards as directed previously.*
2. *Remove excess microplate strips from the plate frame and seal and store them in the original packaging.*
3. *Add 100 µl of the standard, samples, or control per well. Add 100 µl of the sample diluent buffer into the control well (Zero well). At least two replicates of each standard, sample, or control is recommended.*
4. *Cover with the plate sealer provided and incubate for 120 minutes at RT (or 90 min. at 37 °C).*
5. *Remove the cover and discard the liquid in the wells into an appropriate waste receptacle. Invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.*
6. *Add 100 µl of the prepared 1x Biotinylated Anti-Human THBD antibody to each well.*
7. *Cover with plate sealer and incubate for 90 minutes at RT (or 60 minutes at 37°C).*
8. *Wash the plate 3 times with the 1x wash buffer.*
   a. *Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.*
   b. *Add 300 µl of the 1x wash buffer to each assay well. (For cleaner background incubate for 60 seconds between each wash).*
   c. *Repeat steps a-b 2 additional times.*
   d. *Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.*
9. *Add 100 µl of the prepared 1x Avidin-Biotin-Peroxidase Complex into each well. Cover with the plate sealer provided and incubate for 40 minutes at RT (or 30 minutes at 37°C).*
10. *Wash the plate 5 times with the 1x wash buffer.*
    a. *Discard the liquid in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid. It is recommended that the wells are not allowed to completely dry at any time.*
    b. *Add 300 µl of the 1x wash buffer to each assay well. (For cleaner background incubate for 60 seconds between each wash).*
    c. *Repeat steps a-b 4 additional times.*
    d. *Discard the wash buffer in the wells into an appropriate waste receptacle. Then, invert the plate on the benchtop onto a paper towel and tap the plate to gently blot any remaining liquid.*
11. *Add 90 µl of Color Developing Reagent to each well. Cover with the plate sealer provided and incubate in the dark for 30 minutes at RT (or 15-25 minutes at 37°C). (The optimal incubation time must be empirically determined. A guideline to look for is blue shading the top four standard wells, while the remaining standards remain clear.)*
12. *Add 100 µl of Stop Solution to each well. The color should immediately change to yellow.*
13. *Within 30 minutes of stopping the reaction, the O.D. absorbance should be read with a microplate reader at 450nm.*

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Data Analysis

Average the duplicate readings for each standard, sample, and control. Subtract the average zero standard O.D. reading.

It is recommended that a standard curve be created using computer software to generate a four parameter logistic (4-PL) curve-fit. A free program capable of generating a four parameter logistic (4-PL) curve-fit can be found online at: www.myassays.com/four-parameter-logistic-curve.assay. Alternatively, plot the mean absorbance for each standard against the concentration. The measured concentration in the sample can be interpolated by using linear regression of each average relative OD against the standard curve generated using curve fitting software. This will generate an adequate but less precise fit of the data.

For diluted samples, the concentration reading from the standard curve must be multiplied by the dilution factor.

Background on THBD

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 thrombininduced 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) havealso been reported to be associated with atypical hemolytic-uremic syndrome (aHUS).