



EKA52068 - 96 Tests

Human Thrombomodulin Colorimetric Sandwich ELISA Kit

Species Reactivity: Human

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES

Last Updated: March 2023

INSTRUCTION MANUAL

This manual may be updated as a result of continuous improvements.
Please always refer to the hard copy manual included in the kit for your experiment.

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INTRODUCTION

Thrombomodulin is a specific endothelial cell receptor that forms a 1:1 stoichiometric complex with thrombin. This complex is responsible for the conversion of protein C to the activated protein C (protein Ca). Once evolved, protein Ca scissions the activated cofactors of the coagulation mechanism, factor Va and factor VIIIa, and thereby reduces the amount of thrombin generated. Endothelial cells are unique in synthesizing thrombomodulin. Thrombomodulin is N-glycosylated. The iron and 2-oxoglutarate dependent 3-hydroxylation of aspartate and asparagine is (R) stereospecific within EGF domains. Defects in THBD are the cause of thrombophilia due to thrombomodulin defect (THR-THBD), a hemostatic disorder characterized by a tendency to thrombosis. Defects in THBD are a cause of susceptibility to hemolytic uremic syndrome atypical type 6 (AHUS6), an atypical form of hemolytic uremic syndrome. It is a complex genetic disease characterized by microangiopathic hemolytic anemia, thrombocytopenia, renal failure and absence of episodes of enterocolitis and diarrhea. In contrast to typical hemolytic uremic syndrome, atypical forms have a poorer prognosis, with higher death rates and frequent progression to end-stage renal disease. Note: Susceptibility to the development of atypical hemolytic uremic syndrome can be conferred by mutations in various components of or regulatory factors in the complement cascade system. Other genes may play a role in modifying the phenotype.

ASSAY PRINCIPLES

The Human Thrombomodulin ELISA Kit contains the components necessary for quantitative determination of natural or recombinant Human Thrombomodulin concentrations within any experimental sample including cell lysates, serum and plasma. This particular immunoassay utilizes the quantitative technique of a “Sandwich” Enzyme-Linked Immunosorbent Assay (ELISA) where the target protein (antigen) is bound in a “sandwich” format by the primary capture antibodies coated to each well-bottom and the secondary detection antibodies added subsequently by the investigator.

The capture antibodies coated to the bottom of each well are specific for a particular epitope on Human Thrombomodulin while the user-added detection antibodies bind to epitopes on the captured target protein. Amid each step of the procedure, a series of wash steps must be performed to ensure the elimination of non-specific binding between proteins to other proteins or to the solid phase.

After incubation and “sandwiching” of the target antigen, a peroxidase enzyme is conjugated to the constant heavy chain of the secondary antibody (either covalently or via Avidin/Streptavidin-Biotin interactions), allowing for a colorimetric reaction to ensue upon substrate addition. When the substrate TMB (3, 3', 5, 5'-Tetramethylbenzidine) is added, the reaction catalyzed by peroxidase yields a blue color that is representative of the antigen concentration.

Upon sufficient color development, the reaction can be terminated through addition of Stop Solution (2 N Sulfuric Acid) where the color of the solution will turn yellow. The absorbance of each well can then be read by a spectrophotometer, allowing for generation of a standard curve and subsequent determination of protein concentration.

ASSAY FORMAT


Capture Antibody



Capture antibodies specific for the target are coated to the plate. Additional binding sites on the plate are blocked.


Target Antigen



Target antigen present in standard or sample is bound by capture antibodies on the solid-phase.


Biotinylated Detection Antibody



Biotinylated detection antibodies specific for the target are added to bind another epitope on the target antigen.


Streptavidin-HRP



Streptavidin-HRP attaches to detection antibody via high affinity streptavidin-biotin interaction.


Unreacted TMB



TMB substrate is converted to the blue TMB diimine product via the HRP enzyme. Upon addition of acid, the reaction terminates and the wells can be read at 450 nm.


Blue TMB Diimine Product

MATERIALS INCLUDED

Component	Quantity Per Kit	Storage/Stability after first use
Microstrips Coated w/ Capture Antibody	12 x 8-Well Microstrips	1 month at 4°C
Protein Standard	Lyophilized	1 week at 4°C
Biotinylated Detection Antibody	Lyophilized	1 week at 4°C
400x Streptavidin-HRP B	30 µl	1 month at 4°C
Wash Buffer (15x)	50 ml	1 month at 4°C
Assay Diluent NM33	15 ml	1 month at 4°C
Assay Diluent LN28	50 ml	1 month at 4°C
Ready-to-Use Substrate	12 ml	1 month at 4°C
Stop Solution	12 ml	-
Adhesive Plate Sealers	2 Sheets	-
Technical Manual	1 Manual	-

Any unused strips should be rewrapped with plate sealer and placed back into pouch with zipper closed until next use**

ADDITIONAL MATERIALS REQUIRED

The following materials and/or equipment are NOT provided in this kit but are necessary to successfully conduct the experiment:

- Microplate reader able to measure absorbance at 450 nm (with correction wavelength set to 540 nm or 570 nm)
- Micropipettes ranging from 1 µl to 1 ml
- Distilled, deionized, and or purified water (recommended TOC 1-50 ppb, MΩ-cm 18.0)
- Squirt bottle, manifold dispenser, multichannel pipette reservoir or automated microplate washer
- Graph paper or computer software capable of generating or displaying logarithmic functions
- Absorbent paper or vacuum aspirator
- Test tubes or microfuge tubes capable of storing ≥1 ml
- Bench-top centrifuge (optional)
- Bench-top vortex (optional)
- Orbital shaker (optional)

SAMPLE PREPARATION AND STORAGE

Sample Type	Recommended Dilution Factor
Plasma	1 to 10
Serum	1 to 10
Cell Culture Supernatant	1 to 5

Levels of Human Thrombomodulin may vary between samples. Optimal dilution factors for every sample must be determined by the investigator.

Cell Supernatants

Remove large cell components via centrifugation and perform the assay. Dilute cell supernatants at a minimum of 1 to 5 using Assay Diluent LN28 and assay.

Serum

Allow samples to clot in a serum separator tube (SST) for 30 minutes. After sufficient clotting, centrifuge at 1000 x g for 15 minutes and remove serum from SST in preparation for the assay. Dilute serum samples 1 to 10 using Assay Diluent NM33 and incubate 20 mins room temperature (RT) prior to continuing with the assay

Plasma

Use heparin, citrate or EDTA as an anticoagulant to gather plasma from original biological sample. After collection of the plasma, centrifuge for 15 minutes at 1000 x g. This step must be performed within 30 minutes of plasma collection. Dilute plasma samples 1 to 10 using Assay Diluent NM33 and incubate 20 mins prior to RT prior to continuing with the assay.

If samples are to be used within 24 hours, aliquot and store at 4°C. If samples are to be used over a long period of time, aliquot and store between -20°C and -80°C, depending on the duration of storage.

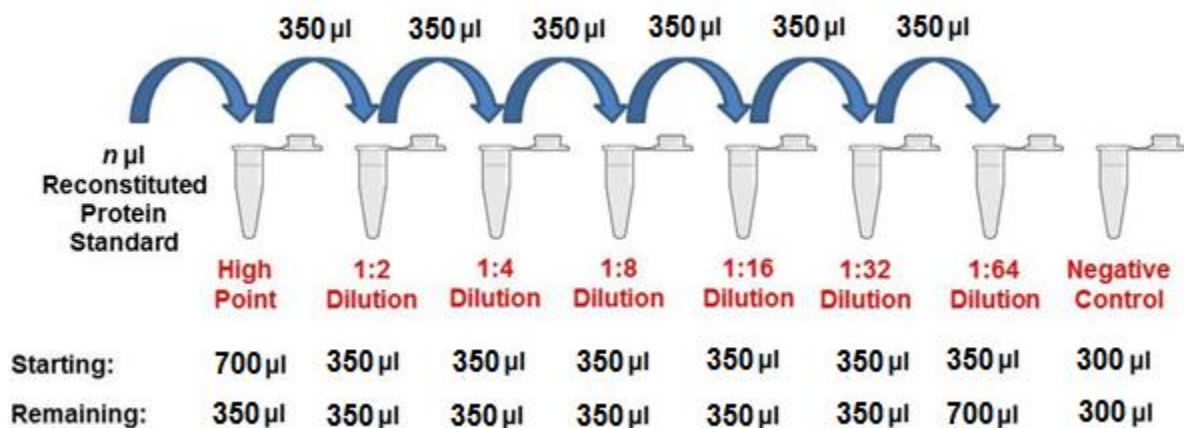
Samples containing a visible precipitate or pellet must be clarified prior to use in the assay.

Avoid repeated freeze/thaw cycles to prevent loss of biological activity of proteins in experimental samples.

REAGENT PREPARATION

All provided solutions should be at ambient temperature prior to use. **We recommend performing the assay in duplicate.** Reagents provided are enough to assay 96 wells and it is recommended to only prepare as much needed on the day of the experiment. All incubation steps should be performed on an orbital shaker to equilibrate solutions when added to the microplate wells.

1. Dilute the 15x Wash Buffer to 1x Wash Buffer using 14 volumes of ddH₂O and 1 volume of 15x Wash Buffer.
2. Reconstitute Detection Antibody with 100 µl of ddH₂O for a concentration of 100x. Mix gently and dilute to 1x prior to use with Assay Diluent LN28.
3. Reconstitute Protein Standard with 83 µl of ddH₂O for a concentration of 60 ng/mL. Mix gently and dilute to the working range of the kit, 32-2000 pg/mL. To obtain a 7 point 2 fold dilution for the standard curve 23.33 µl of standard into 676.67 µl of diluent.
 - a. Dilute Protein Standard with Assay Diluent NM33 when experimenting with Serum/Plasma samples. **OR.**
 - b. Dilute Protein Standard with Assay Diluent LN28 when experimenting with Cell Supernatant samples.



4. Mix the 400x Streptavidin-HRP B (SAV-HRP) gently. Dilute to 1x using Assay Diluent 1TD.
5. Stop Solution and Ready to Use Substrate are ready to use and do not need dilution.



ASSAY PROCEDURE

1. Prepare all reagents to working concentrations, standards to desired range, and samples to appropriate dilution factors.
2. Remove desired number of capture antibody coated strips for experiment and place remaining strips back into dry pouch with desiccant for 4°C storage.
3. Add 100 µl of Standards/ Samples to each well and incubate on orbital shaker at room temperature (RT) for 2 hrs.
4. Aspirate the solution and add 300 µl of 1x Wash Buffer to each well being used and gently shake for 2-3 mins on an orbital shaker. Repeat this process 3 times. After the last wash ensure no liquid remains by inverting the plate and tapping it against clean paper towels.
5. Add 100 µl of 1x Detection Antibody to each well and incubate for 2 hrs. on an orbital shaker at RT.
6. Repeat step 4.
7. Add 100 µl of 1x SAV-HRP B to each well and incubate for 30 mins on an orbital shaker at RT
8. Repeat step 4.
9. Add 100 µl of Ready to use Substrate to each well, cover plate from light, and incubate for 20 mins on an orbital shaker at RT.
10. Add 100 µl of Stop Solution to each well and read at 450 nm.

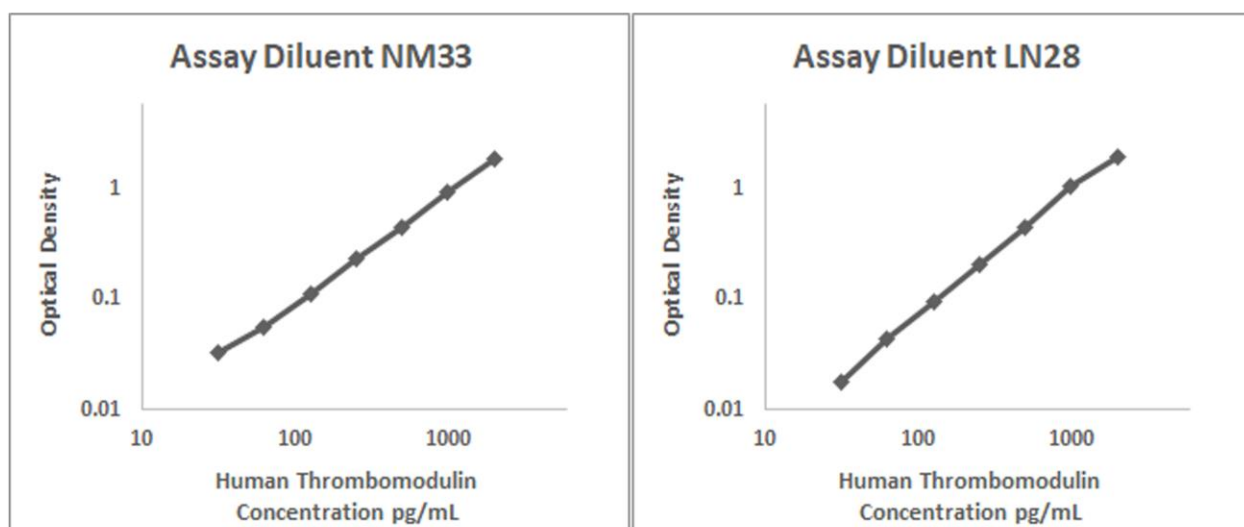
DATA ANALYSIS

Average the duplicate or triplicate readings for each standard, control and sample and subtract the average zero standard optical density.

Generate a standard curve by using Microsoft Excel or other computer software capable of establishing a 4-Parameter Logistic (4-PL) curve fit. If using Excel or an alternative graphing tool, plot the average optical density values in absorbance units (y-axis) against the known standard concentrations in pg/ml (x-axis).

Only use the values in which a noticeable gradient can be established. Afterwards, generate a best fit curve or “trend-line” through the plotted points via regression analysis.

TYPICAL DATA



Concentration pg/ml	2000.00	1000.00	500.00	250.00	125.00	62.50	31.25
Average Optical Density NM33	1.90	0.94	0.45	0.24	0.11	0.06	0.03
Average Optical Density LN28	1.97	1.05	0.46	0.21	0.09	0.04	0.02

The data and subsequent graph were obtained after performing a cytokine ELISA for Human Thrombomodulin. Each known sample concentration was assayed in triplicate.

The standard curves shown are for demonstration. A new curve must be generated for each experiment.

RECOVERY

Three concentrations of recombinant Human Thrombomodulin was spiked into various samples to measure the recovery percentage of recombinant protein in duplicate.

Sample Type	Average Recovery %	Range %
Plasma (10%)	95	82-105
Serum (10%)	104	93-113
RPMI 10% FBS (20%)	105	104-108

LINEARITY

Human Thrombomodulin recombinant protein was spiked into various biological samples and serial diluted with Assay Diluent NM33 for serum samples and Assay Diluent LN28 for RPMI 10% FBS.

		EDTA Plasma	Serum	RPMI 10% FBS
Neat	pg/ml	242.87	261.37	270.84
	Expected %	97	105	108
1 to 2	pg/ml	115.68	114.43	136.13
	Expected %	92	93	109
1 to 4	pg/ml	71.04	67.66	65.51
	Expected %	108	108	105
1 to 8	pg/ml	33.28	32.28	34.39
	Expected %	103	103	110



SENSITIVITY

The Human Thrombomodulin ELISA Kit allows for the detection and quantification of endogenous levels of natural and/or recombinant Human Thrombomodulin proteins within the range of 32-2000 pg/ml.

CROSS REACTIVITY AND SPECIFICITY

The Human Thrombomodulin ELISA is capable of recognizing both recombinant and naturally produced Human Thrombomodulin proteins. The antigens listed below were tested at 50 ng/ml and did not exhibit significant cross reactivity or interference.

- Human: Coagulation Factor II, Coagulation Factor VII, Coagulation Factor X, Coagulation Factor XI
- Murine: Thrombomodulin

SUMMARIZED PROTOCOL

Reconstitute Biotinylated Detection Antibody and Protein Standard and dilute the 15x Wash Buffer as specified.



Perform serial dilution of Protein Standard and prepare samples as desired. See sample preparation section for instructions to dilute serum and plasma samples.



Add 100ul of Protein Standard, sample or control to each well and incubate for 2 hours at room temperature.



Aspirate Protein Standards, samples or controls out and wash plate 4 times.



Dilute Biotinylated Detection Antibody as specified. Add 100ul to each well and incubate for 2 hours at room temperature.



Aspirate Biotinylated Detection Antibody out and wash plate 4 times.



Dilute 400x Streptavidin-HRP as specified. Add 100ul of 1x Streptavidin-HRP to each well and incubate at room temperature for 30 minutes.



Aspirate 1x Streptavidin-HRP out and wash plate 4 times.



Add 100ul of Ready-to-Use Substrate to each well and incubate at room temperature for color development.



Add 100ul of Stop Solution and read plate at 450nm.

HEALTH AND SAFETY PRECAUTIONS

- Reagents provided in this kit may be harmful if ingested, inhaled or absorbed through the skin. Please carefully review the MSDS for each reagent before conducting the experiment.
- Stop Solution contains 2 N Sulfuric Acid (H_2SO_4) and is an extremely corrosive agent. Please wear proper eye, hand and face protection when handling this material. When the experiment is finished, be sure to rinse the plate with copious amounts of running water to dilute the Stop Solution prior to disposing the plate.

ASSAY RESTRICTIONS

- This ELISA kit is intended for research purposes only, NOT diagnostic or clinical procedures of any kind.
- Materials included in this kit should NOT be used past the expiration date on the kit label.
- Reagents or substrates included in this kit should NOT be mixed or substituted with reagents or substrates from any other kits.
- Variations in pipetting technique, washing technique, operator laboratory technique, kit age, incubation time or temperature may cause differences in binding affinity of the materials provided.
- The assay is designed to eliminate interference and background by other cellular macromolecules or factors present within any biological samples. However, the possibility of background noise cannot be fully excluded until all factors have been tested using the assay kit.
- Individual results may vary due to differences in technique, plasticware and water sources.

ELISA PLATE TEMPLATE

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TECHNICAL SUPPORT

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