

ab47358 – Thrombomodulin Human ELISA Set

Instructions for Use

For the quantitative measurement of Human thrombomodulin in cell culture supernatants, buffered solutions, serum, plasma and other body fluids.

This product is for research use only and is not intended for diagnostic use.

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1. BACKGROUND

Abcam's thrombomodulin Human *in vitro* ELISA Set is designed for the quantitative measurement of thrombomodulin in cell culture supernatants, buffered solutions, serum, plasma and other body fluids.

An antibody specific for thrombomodulin is coated onto the wells of the microtiter plates. Samples, including standards of known thrombomodulin concentrations and unknowns are pipetted into these wells and incubated at room temperature. The wells are then washed and a biotinylated antibody specific for thrombomodulin is added to the wells and incubated. After further washing, a Streptavidin-peroxydase conjugate is added to each well, and incubated. The wells are then washed to remove all unbound enzyme and TMB solution, which actab47358100s on the bound enzyme is added to induce a colored reaction product. The intensity of this colored product is directly proportional to the concentration of thrombomodulin present in the samples.

Thrombomodulin, also known as CD141 or BDC-A is an transmembrane protein expressed predominantly on the surface of vascular endothelial cells, but also on other cell types such as macrophages, monocytes, subpopulations of myeloid dendritic cells and keratinocytes. Thrombomodulin is a type 1 membrane glycoprotein of 74kDa consisting on a single chain with 6 Epidermal Growth Factor (EGF)-like domains critical for Thrombin and protein C binding, and four other domains (an N-terminal C-type lectin domain, a highly glycosylated serine/threonine-rich domain, a transmembrane segment, and a short C-terminal cytoplasmic tail. It acts as an important component in the anti-coagulation and fibrinolysis system: cofactor for active thrombin, the thrombomodulin-thrombin complex activates protein C to degrade coagulation factors, inhibiting coagulation and fibrinolysis. When vascular endothelial cells injury occurs, proteolytic cleavage release multiple fragments of thrombomodulin in circulation, so it represents a good marker for endothelial cell damage in

pathologies such as cardiovascular disease, acute coronary syndrome, pulmonary thromboembolism or severe hemorrhage. Thrombomodulin has been described as an anticoagulant effect protein, and its therapeutic potential for preventing deep vein thrombosis and its use in the treatment of disseminated intravascular coagulation has been demonstrated in preliminary data. More recently, other biological effects beyond anticoagulation have been demonstrated such as an anti-inflammatory, cell adhesion and proliferation regulation. It has also been demonstrated that thrombomodulin may be able to suppress the metastatic capacity of tumor cells, and hence have a protective role in many types of malignancies. This kit will recognize both endogenous and recombinant Human thrombomodulin.

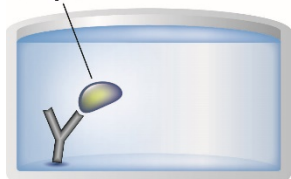
2. ASSAY SUMMARY

Primary Capture Antibody



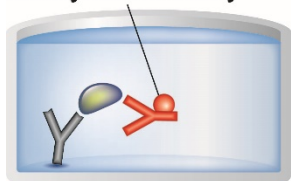
Remove appropriate number of antibody coated well strips. Equilibrate all reagents to room temperature. Prepare all the reagents, samples, and standards as instructed.

Sample



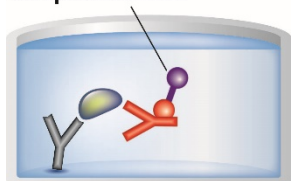
Add standard or sample to each well used.

Biotinylated Antibody



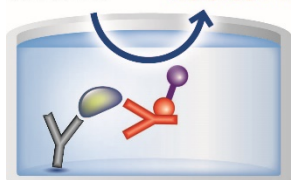
Add biotinylated labeled detector antibody to each well used. Incubate at room temperature.

Streptavidin-HRP



Aspirate and wash each well. Add prepared Streptavidin-HRP mix to each well. Incubate at room temperature.

Substrate Colored Product



Aspirate and wash each well. Add the TMB Solution to each well until color develops and then add the Stop Solution. Immediately begin recording the color development.

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at +2-8°C immediately upon receipt.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 9. Reagent Preparation.

5. MATERIALS SUPPLIED

Item	Quantity			Storage Condition (Before Preparation)
	5 x 96 tests	10 x 96 tests	15 x 96 tests	
Thrombomodulin Capture antibody	1 x 100 µL	2 x 100 µL	3 x 100 µL	+2-8°C
Detection biotinylated anti-Thrombomodulin antibody	1 x 1 vial	2 x 1 vial	3 x 1 vial	+2-8°C
Thrombomodulin standard	5 x 1 vial	10 x 1 vial	15 x 1 vial	+2-8°C
Streptavidin HRP	1 x 1 vial	2 x 1 vial	3 x 1 vial	+2-8°C
Ready-to-Use TMB	2 x 1 vial	4 x 1 vial	6 x 1 vial	+2-8°C

6. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 96 well Microtitre plates.
- Reconstitution Buffer
1X PBS + 0.09% Azide. Once prepared store at 2-8°C for up to one week.
- Coating Buffer
1X PBS, pH 7.2-7.4. Once prepared store at 2-8°C for up to one week.
- Wash Buffer
1X PBS + 0.05% Tween20. Once prepared use immediately.
- Blocking Buffer
1X PBS + 5% BSA. Once prepared store at 2-8°C for up to one week.
- Standard and Secondary Antibody Dilution Buffer
1X PBS + 1% BSA. Once prepared store at 2-8°C for up to one week.
- HRP Diluent Buffer
1X PBS + 1% BSA + 0.1% Tween20. Once prepared store at 2-8°C for up to one week.
- Stop Reagent (1M Sulfuric Acid).
- Microtitre plate reader with appropriate filters (450 nm required with optional 620 nm reference filter).
- Microplate washer or wash bottle.
- 10, 50, 100, 200 and 1,000 μ L adjustable single channel micropipettes with disposable tips.
- 50-300 μ L multi-channel micropipette with disposable tips.
- Multichannel micropipette reagent reservoirs.
- Distilled water.

- Vortex mixer.
- Miscellaneous laboratory plastic and/or glass, if possible sterile.

7. LIMITATIONS

- Exact conditions may vary from assay to assay, a standard curve should be generated for every assay performed.
- Bacterial or fungal contamination of either samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Completely empty wells before dispensing fresh 1X Wash Buffer. Do not allow wells to sit uncovered or dry for extended periods.
- Do not extrapolate the standard curve beyond the maximum standard curve point. The dose-response is non-linear in this region and good accuracy is difficult to obtain. Concentrated samples above the maximum standard concentration must be diluted with Standard diluent or with your own sample buffer to produce an OD value within the range of the standard curve. Following analysis of such samples always multiply results by the appropriate dilution factor to produce actual final concentration.

8. TECHNICAL HINTS

- Kit components should be stored as indicated. All the reagents should be equilibrated to room temperature before use. Reconstituted standards should be discarded after use.
- Use a clean disposable plastic pipette tip for each reagent, standard, or specimen addition in order to avoid cross-contamination; for the dispensing of the Stop Solution and substrate solution, avoid pipettes with metal parts.
- Thoroughly mix the reagents and samples before use by agitation or swirling.
- All residual washing liquid must be drained from the wells by efficient aspiration or by decantation followed by tapping the plate forcefully on absorbent paper. Never insert absorbent paper directly into the wells.
- The TMB solution is light sensitive. Avoid prolonged exposure to light. Also, avoid contact of the TMB solution with metal to prevent color development. Warning TMB is toxic avoid direct contact with hands. Dispose of properly.
- If a dark blue color develops within a few minutes after preparation, this indicates that the TMB solution has been contaminated and must be discarded.
- Read absorbances within 1 hour after completion of the assay.
- When pipetting reagents, maintain a consistent order of addition from well-to-well. This will ensure equal incubation times for all wells.
- Dispense the TMB solution within 15 minutes following the washing of the microtiter plate.

This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. Please contact our Technical Support staff with any questions.

9. REAGENT PREPARATION

Equilibrate all reagents and samples to room temperature (18-25°C) prior to use.

9.1 Capture Antibody

For 1 x 96 well plate add 20 µL of Capture Antibody into 10 mL of Coating Buffer.

9.2 Reconstituted Biotinylated anti Thrombomodulin Detection Antibody

The antibody is prepared immediately before use. Reconstitute each vial with 550 µL PBS + 0.1% Azide w/v or another preservative. Dilute the reconstituted biotinylated anti-Thrombomodulin with the Standard and Secondary Antibody Dilution Buffer in an appropriate clean glass vial. For 1 x 96 well plate add 100 µL of the reconstituted Detection Antibody into 5 mL of Standard and Secondary Antibody Dilution Buffer. Once prepared store at 2-8°C for up to one year.

9.3 Preparation of Streptavidin-HRP

It is recommended to centrifuge the vial for a few seconds in a microcentrifuge to collect all the volume at the bottom of the vial. Dilute 5 µL of Streptavidin-HRP into 500 µL of HRP Diluent Buffer **immediately before use**. For 1 x 96 well plate take 150 µL of the diluted HRP solution into 10 mL of HRP Diluent Buffer. Do-not keep these solutions for future experiments.

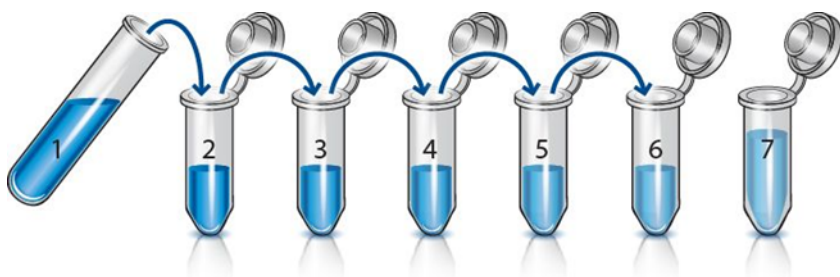
10. STANDARD PREPARATION

- Standard vials must be reconstituted with the volume of standard dilution buffer shown on the vial immediately prior to use.
 - 10.1. Reconstitute the Thrombomodulin standard sample by adding the volume of Standard Dilution Buffer indicated on the vial label by pipette. Mix thoroughly and gently. This is the 20 ng/mL **Standard #1** Solution (see table below).
Note: The reconstituted Standard #1 should be discarded after use and not stored for reuse.
 - 10.2. Label six tubes with Standards # 2 – 7.
 - 10.3. Add 300 μ L Standard Dilution Buffer into Standards # 2 - 6 and 500 μ L Standard Dilution Buffer into Standard #7.
 - 10.4. Prepare Standard #2 by transferring 300 μ L from Standard #1 to Standard #2. Mix thoroughly and gently.
 - 10.5. Prepare Standard #3 by transferring 300 μ L from Standard #2 to Standard #3. Mix thoroughly and gently.
 - 10.6. Using the table below as a guide, repeat for Standards #4 through to Standard #6.
 - 10.7. Standard #7 contains no protein and is the Blank control.

ASSAY PREPARATION

Standard Dilution Preparation Table

Standard #	Sample to Dilute	Volume to Dilute (μL)	Volume of Diluent (μL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)
1	See Step 10.1				20
2	Standard #1	300	300	20	10
3	Standard #2	300	300	10	5
4	Standard #3	300	300	5	2.5
5	Standard #4	300	300	2.5	1.25
6	Standard #5	300	300	1.25	0.625
7 (Blank)	-	-	500	0	0



11. SAMPLE PREPARATION AND STORAGE

- Cell culture supernatants, Human serum, plasma or other biological samples will be suitable for use in the assay.
- If not analyzed shortly after collection, samples should be aliquoted (250-500 μ L) to avoid repeated freeze-thaw cycles and stored frozen at -70°C . Avoid multiple freeze-thaw cycles of frozen specimens.
- Do not thaw samples by heating at 37°C or 56°C . Thaw at room temperature and make sure that sample is completely thawed and homogeneous before use. When possible avoid use of badly haemolysed or lipemic sera. If large amounts of particles are present these should be removed prior to use by centrifugation or filtration.

11.1. Cell culture supernatants

Remove particulates and aggregates by centrifuging at approximately 1,000 x g for 10 minutes.

11.2. Serum

Use pyrogen/endotoxin free collecting tubes. Serum should be removed rapidly and carefully from the red cells after clotting. Following clotting, centrifuge at approximately 1,000 x g for 10 minutes and remove serum.

11.3. Plasma

EDTA, citrate and heparin plasma can be assayed. Centrifuge samples at 1,000 x g for 30 minutes to remove particulates. Harvest plasma.

12. PLATE PREPARATION

- If you wish to store the coated and blocked plates for future use, bench dry each plate at room temperature (18 to 25°C) for 24 hours. Cover the plates and then store at 2-8°C in a sealed plastic bag with desiccant for up to 12 months.
- 12.1. Add 100 µL of diluted Capture Antibody to each well. Cover with a plastic plate cover and incubate at 4°C overnight.
- 12.2. Remove the cover and wash the plate as follows:
 - 12.2.1. Aspirate the liquid from each well.
 - 12.2.2. Dispense 400 µL of washing solution into each well.
 - 12.2.3. Aspirate the contents of each well.
 - 12.2.4. Repeat step 12.2.2 and 12.2.3.
- 12.3. Add 250 µL of Blocking Buffer to each well.
- 12.4. Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 2 hours.
- 12.5. Remove the cover and wash the plate as follows:
 - 12.5.1. Aspirate the liquid from each well.
 - 12.5.2. Dispense 400 µL of washing solution into each well.
 - 12.5.3. Aspirate the contents of each well.
 - 12.5.4. Repeat step 12.5.2 and 12.5.3 two times.

13. ASSAY PROCEDURE

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**
- **We strongly recommend that every vial is mixed thoroughly without foaming prior to use except the standard vial which must be mixed gently by inversion only.**
- **Note: Final preparation of biotinylated anti-thrombomodulin and Streptavidin-HRP should occur immediately before use.**

13.1 Prepare Standard curve as shown in Section 10.

13.2 Add 100 μ L of each standard or sample to appropriate wells in duplicate.

13.3 Add 50 μ L of diluted Detection Antibody into all wells.

13.4 Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 1 hour.

13.5 Remove the cover and wash the plate as follows:

13.5.1 Aspirate the liquid from each well

13.5.2 Dispense 400 μ L of washing solution into each well

13.5.3 Aspirate the contents of each well

13.5.4 Repeat step 13.5.2 and 13.5.3.

13.6 Add 100 μ L of Streptavidin-HRP solution into all wells.

13.7 Cover with a plastic plate cover and incubate at room temperature (18 to 25°C) for 30 minutes.

13.8 Repeat wash step 13.5.

13.9 Add 100 μ L of ready-to-use TMB Substrate Solution into all wells.

13.10 Incubate in the dark for 5-15 minutes* at room temperature. Avoid direct exposure to light by wrapping the plate in aluminium foil.

**Incubation time of the substrate solution is usually determined by the ELISA reader performance. Many ELISA readers only record absorbance up to 2.0 O.D. Therefore the colour development within individual microwells must be observed by the analyst, and the substrate reaction stopped before positive wells are no longer within recordable range.*

- 13.11 Add 100 μ L of Stop Reagent into all wells.
- 13.12 Read the absorbance value of each well (immediately after step 13.11.) on a spectrophotometer using 450 nm as the primary wavelength and optionally 620 nm as the reference wave length (610 nm to 650 nm is acceptable).

14. CALCULATIONS

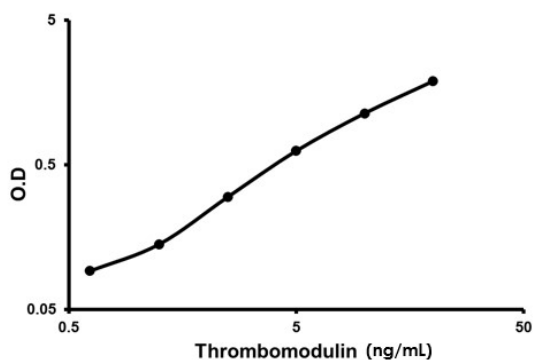
Calculate the average absorbance values for each set of duplicate standards and samples. Ideally duplicates should be within 20% of the mean.

Generate a linear standard curve by plotting the average absorbance of each standard on the vertical axis versus the corresponding thrombomodulin standard concentration on the horizontal axis.

The amount of thrombomodulin in each sample is determined by extrapolating OD values against thrombomodulin standard concentrations using the standard curve.

15. TYPICAL DATA

TYPICAL STANDARD CURVE - Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.



Conc. (pg/mL)	O.D.
0	0.038
0.62	0.092
1.25	0.141
2.5	0.299
5	0.622
10	1.132
20	1.889

Figure 1. Example of typical Thrombomodulin standard curve data.

16. ASSAY SPECIFICITY

The assay recognizes natural Human thrombomodulin. To define specificity of this thrombomodulin antibody pair, several proteins were tested for cross reactivity using the Abcam thrombomodulin pre-coated ELISA kit (which contains the same antibodies). There was no cross reactivity observed for any protein tested (IL-1 α , IL-1 β , IL-2, IL-17A, IFN γ , IL-17F, CD116, CD54, CD138, and ICAM-2).

17. ASSAY SENSITIVITY

The sensitivity, minimum detectable dose of this thrombomodulin antibody pair was determined using the Abcam thrombomodulin ELISA kit (which contains the same antibodies) and was found to be <0.31 ng/mL. This was determined by adding 2 standard deviations to the mean OD obtained when the zero standard was assayed in 6 independent experiments.

18. TROUBLESHOOTING

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standards dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Incubation times too brief	Ensure sufficient incubation times; change to overnight standard/sample incubation
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Plate is insufficiently washed	Review manual for proper wash technique. If using a plate washer, check all ports for obstructions
	Contaminated wash buffer	Prepare fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store the reconstituted protein at -80°C, all other assay components +2-8°C. Keep substrate solution protected from light.

19. NOTES

For all technical and commercial enquires please go to:

www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)