Quantikine® ELISA

Mouse Thrombomodulin/BDCA-3 Immunoassay

Catalog Number MTHBD0

For the quantitative determination of mouse Thrombomodulin concentrations in cell culture supernates, serum, plasma, and urine.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Thrombomodulin, also known as BDCA-3 and CD141, is an approximately 75 kDa cell surface protein that plays important roles in inflammation and blood coagulation (1, 2). Mature mouse Thrombomodulin consists of a 501 amino acid (aa) extracellular domain (ECD) with a N-terminal C-type lectin domain and six EGF-like domains, a 24 aa transmembrane segment, and a 36 aa cytoplasmic domain (3). Within the ECD, mouse Thrombomodulin shares 66% and 85% aa sequence identity with human and rat Thrombomodulin, respectively. It is variably glycosylated with N-linked and O-linked glycans as well as chondroitin sulfate glycosaminoglycans (1, 4). Thrombomodulin is expressed on vascular endothelial cells (EC), arterial smooth muscle cells, monocytes, and macrophages (5-7). It binds and regulates the activity of Thrombin, a central activator of blood coagulation (8). It enhances the Thrombin-mediated activation of the anti-coagulant Protein C and the anti-fibrinolytic TAFI/Carboxypeptidase B2 (9). Thrombomodulin also inhibits the ability of Thrombin to activate several pro-coagulant proteins (*e.g.* Fibrinogen, Factor V, Factor XIII, PAR-1) (1).

Thrombomodulin exerts multiple anti-inflammatory effects and reduces the severity of disease involving chronic inflammation (10-13). Its lectin domain mediates binding to Lewis Y carbohydrates on vascular endothelial cells, resulting in inhibition of leukocyte extravasation to sites of inflammation (11, 14). It also interferes with complement activation and deposition (13). On monocytes and macrophages, Thrombomodulin is a component of the CD14-TLR4 complex which enables inflammatory responsiveness to LPS and also the clearance of Gram negative bacteria (14, 15). In addition, Thrombomodulin decreases the pro-inflammatory effect of the alarmin HMGB1 by sequestering it and enhancing its Thrombin-mediated inactivation (16, 17).

Soluble fragments of Thrombomodulin circulate in the serum in coagulation disorders, inflammation, and organ failure (18). They can reduce tissue damage following ischemia/reperfusion (7, 12) and can differentially regulate angiogenesis (6, 19, 20). Various soluble forms of Thrombomodulin, which are also present in the urine and rheumatoid arthritis synovial fluid, retain their ability to coactivate Protein C and inhibit coagulation (21-23). In cancer, Thrombomodulin suppresses tumor angiogenesis, growth, and epithelial-mesenchymal transition, although isolated domains can modulate these effects (19, 24-26).

The Quantikine Mouse Thrombomodulin/BDCA-3 immunoassay is a 4.5 hour solid-phase ELISA designed to measure Thrombomodulin in mouse cell culture supernates, serum, plasma, and urine. It contains NS0-expressed recombinant mouse Thrombomodulin and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant mouse Thrombomodulin. Results obtained using natural mouse Thrombomodulin showed dose response curves that were parallel to the standard curves obtained using the Quantikine mouse kit standards. These results indicate that this kit can be used to determine relative mass values for natural mouse Thrombomodulin.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for mouse Thrombomodulin has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any Thrombomodulin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse Thrombomodulin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of Thrombomodulin bound in the initial step. The sample values are then read off the standard curve.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, further dilute samples with Calibrator Diluent and repeat the assay.
- Any variation in standard diluent, operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- Variations in sample collection, processing, and storage may cause sample value differences.
- This assay is designed to eliminate interference by other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

TECHNICAL HINTS

- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the Substrate Solution. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.

MATERIALS PROVIDED & STORAGE CONDITIONS

Store the unopened kit at 2-8 °C. Do not use past kit expiration date.

PART	PART #	DESCRIPTION	STORAGE OF OPENED/ RECONSTITUTED MATERIAL
Mouse Thrombodmodulin Microplate	894689	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse Thrombomodulin.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of the zip-seal. May be stored for up to 1 month at 2-8 °C.*
Mouse Thrombodmodulin Conjugate	894690	12 mL of a polyclonal antibody specific for mouse Thrombomodulin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse Thrombodmodulin Standard	894691	Recombinant mouse Thrombomodulin in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Mouse Thrombodmodulin Control	894692	Recombinant mouse Thrombomodulin in a buffered protein base with preservatives; lyophilized. The assay value of the Control should be within the range specified on the label.	
Assay Diluent RD1-38	895301	12 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5T	895175	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative. *May turn yellow over time.*	
Color Reagent A	895000	12 mL of stabilized hydrogen peroxide.	
Color Reagent B	895001	12 mL of stabilized chromogen (tetramethylbenzidine).	
Stop Solution	895174	23 mL of diluted hydrochloric acid.	
Plate Sealers	N/A	4 adhesive strips.	

* Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 500 mL graduated cylinder.
- Test tubes for dilution of standards and samples.

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution.

Some components in this kit contain ProClin® which may cause an allergic skin reaction. Avoid breathing mist.

Color Reagent B may cause skin, eye, and respiratory irritation. Avoid breathing fumes.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Please refer to the MSDS on our website prior to use.

SAMPLE COLLECTION & STORAGE

The sample collection and storage conditions listed below are intended as general guidelines. Sample stability has not been evaluated.

Cell Culture Supernates - Remove particulates by centrifugation. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge for 20 minutes at 2000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Citrate plasma has not been validated for use in this assay.*

Urine - Collect urine using a metabolic cage. Remove any particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Centrifuge again before assaying to remove any additional precipitates that may appear after storage.

SAMPLE PREPARATION

Serum, plasma, and urine samples require a 10-fold dilution. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent RD5T.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

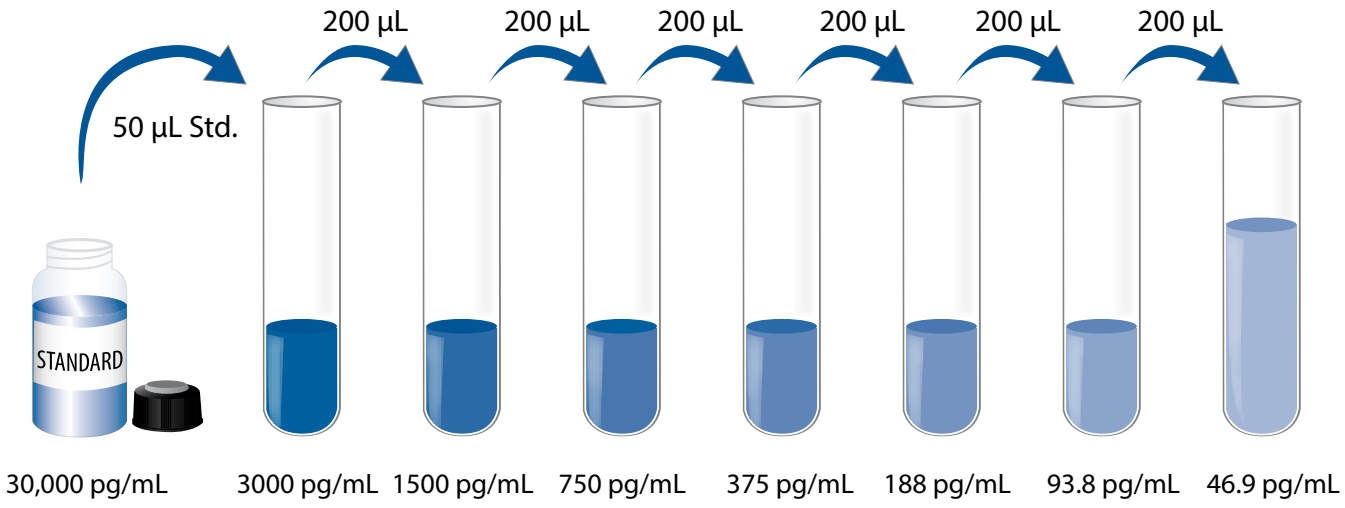
Mouse Thrombomodulin Control - Reconstitute the Control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the Control undiluted.

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Mouse Thrombomodulin Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse Thrombomodulin Standard with deionized or distilled water. This reconstitution produces a stock solution of 30,000 pg/mL. Allow the stock solution to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 450 µL of Calibrator Diluent RD5T into the 3000 pg/mL tube. Pipette 200 µL into the remaining tubes. Use the stock solution to produce a 2-fold dilution series (below). Mix each tube gently but thoroughly before the next transfer. The 3000 pg/mL standard serves as the high standard. Calibrator Diluent RD5T serves as the zero standard (0 pg/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all standards, control, and samples be assayed in duplicate.

1. Prepare all reagents, standard dilutions, control, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 50 µL of Assay Diluent RD1-38 to each well.
4. Add 50 µL of Standard, Control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
6. Add 100 µL of Mouse Thrombomodulin Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.
7. Repeat the aspiration/wash as in step 5.
8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the benchtop. **Protect from light.**
9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

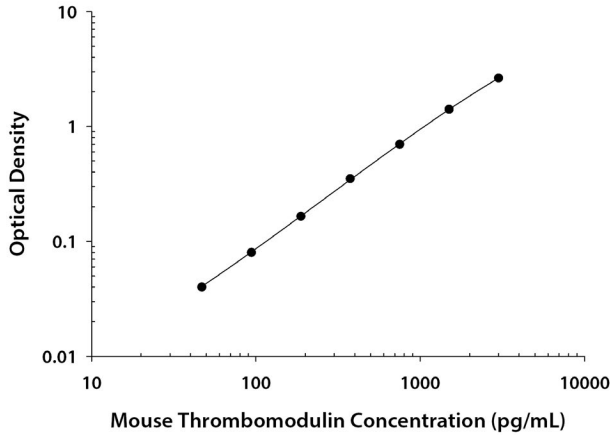
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the mouse Thrombomodulin concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis. This procedure will produce an adequate but less precise fit of the data.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

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



(pg/mL)	O.D.	Average	Corrected
0	0.040 0.046	0.043	—
46.9	0.081 0.084	0.083	0.040
93.8	0.122 0.124	0.123	0.080
188	0.207 0.208	0.208	0.165
375	0.388 0.400	0.394	0.351
750	0.731 0.751	0.741	0.698
1500	1.397 1.504	1.451	1.408
3000	2.632 2.716	2.674	2.631

PRECISION

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

Inter-assay Precision (Precision between assays)
Three samples of known concentration were tested in twenty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of kit components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	108	388	1159	121	380	1155
Standard deviation	6.67	16.0	44.8	16.0	38.1	136
CV (%)	6.2	4.1	3.9	13.2	10.0	11.8

RECOVERY

The recovery of mouse Thrombomodulin spiked to levels throughout the range of the assay was evaluated.

	Average % Recovery	Range
Cell culture media (n=4)	104	91-115%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of mouse Thrombomodulin in each matrix were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay. All samples were diluted prior to assay.

		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	103	98	99	99	104
	Range (%)	98-106	95-104	98-100	97-100	102-106
1:4	Average % of Expected	106	98	97	99	110
	Range (%)	99-111	90-101	94-98	96-100	106-112
1:8	Average % of Expected	111	98	94	101	108
	Range (%)	104-119	84-102	89-98	99-102	99-113
1:16	Average % of Expected	109	92	89	98	104
	Range (%)	103-114	89-100	82-96	96-103	96-112

SENSITIVITY

Twenty-eight assays were evaluated and the minimum detectable dose (MDD) of mouse Thrombomodulin ranged from 2.37-19.1 pg/mL. The mean MDD was 5.82 pg/mL.

The MDD was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

This immunoassay is calibrated against a highly purified NS0-expressed recombinant mouse Thrombomodulin produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Urine - Samples were evaluated for the presence of mouse Thrombomodulin in this assay.

Samples	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Serum (n=10)	21,660	15,370-29,404	4555
EDTA plasma (n=5)	14,784	11,730-17,740	2192
Heparin plasma (n=5)	19,383	17,249-24,810	3090
Urine (n=9)	15,749	9100-24,490	5396

Cell Culture Supernates - Lungs from mice were rinsed with PBS then homogenized with a tissue homogenizer and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 3 days. An aliquot of the cell culture supernate was removed, assayed for mouse Thrombomodulin, and measured 15,860 pg/mL.

SPECIFICITY

This assay recognizes natural and recombinant mouse Thrombomodulin.

The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range mouse Thrombomodulin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:

Coagulation Factor VII
Coagulation Factor Xa
Coagulation Factor XI
Coagulation Factor XIV
Protein S

Recombinant human:

Coagulation Factor VII
Coagulation Factor Xa
Coagulation Factor XI
Coagulation Factor XIV
Protein S
Thrombin
Thrombomodulin

Natural proteins:

Coagulation Factor II

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