PRODUCT INFORMATION & MANUAL

Human Thrombomodulin/BDCA-3 Valukine™ ELISA

Catalog Number: VAL252

For the quantitative determination of natural and recombinant human Thrombomodulin concentrations

For research use only.
Not for diagnostic or therapeutic procedures.

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Please refer to the kit label for expiry date.
Novus kits are guaranteed for 3 months from date of receipt

Version 202411.1

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I. BACKGROUND

Human Thrombomodulin, also known as BDCA-3 and CD141, is a transmembrane protein mainly expressed by vascular endothelial cells. Thrombomodulin is an important component in the anti-coagulation and fibrinolysis system. When the coagulation cascade is activated, prothrombin is converted to thrombin by coagulation factors Va and VIIIa, ultimately leading to fibrin clot formation. Thrombomodulin functions as a cell surface receptor for thrombin. The thrombomodulin-thrombin complex activates protein C to degrade coagulation factors Va and VIIIa, thereby reducing the amount of thrombin generated and inhibiting coagulation. Thrombomodulin-thrombin complex also activates thrombin-activatable fibrinolysis inhibitor (TAFI), creating a carboxypeptidase that inhibits fibrinolysis (1-3).

Thrombomodulin is a type I transmembrane protein, consisting of an N-terminal C-type lectin domain, six contiguous epidermal growth factor (EGF)-like domains, a highly glycosylated serine/threonine-rich domain, a transmembrane segment, and a short C-terminal cytoplasmic tail. The EGF-like domains are critical for thrombin and protein C binding (4). When vascular endothelial cell injury occurs, the extracellular domain of thrombomodulin is released from the cell surface by proteolytic cleavage, giving rise to multiple fragments in the circulation (5, 6). Quantification of soluble thrombomodulin in the circulation therefore provides insight into the extent of vascular endothelial cell damage. It has been documented that patients suffering from cardiovascular diseases, such as acute coronary syndrome, pulmonary thromboembolism, and severe hemorrhage, have increased circulating thrombomodulin concentrations (7-9). Soluble thrombomodulin retains anticoagulant effect and its therapeutic potential has been explored. Preliminary data has shown that it might have value in preventing deep vein thrombosis and in treatment of disseminated intravascular coagulation (10-12).

Thrombomodulin also contributes to other biological processes beyond anticoagulation. It is well established that the protein C system as well as the thrombin-protease activated receptor system are important coagulation factors that play important roles in inflammation. By modulating the activity of these mediators, thrombomodulin exhibits an anti-inflammatory effect (13-14). Thrombomodulin also regulates cell adhesion and proliferation through its lectin-binding domain (15, 16). Additionally, since mouse embryos lacking functional thrombomodulin cannot survive, thrombomodulin might have unique functions during development (17, 18). Furthermore, accumulating experimental evidence has demonstrated that thrombomodulin may be able to suppress the metastatic capacity of tumor cells to confer a protective role in many types of malignancies (19).

II. OVERVIEW

A. PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. An antibody specific for human Thrombomodulin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any human Thrombomodulin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked antibody specific for human Thrombomodulin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, TMB substrate (Chromogenic agent) is added to the wells and color develops in proportion to the amount of human Thrombomodulin bound in the initial step. The color development is stopped, and the intensity of the color is measured.

B. LIMITATIONS OF THE PROCEDURE

- **FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**
- This kit is suitable for cell culture supernates, cell lysates, human serum, human plasma and human urine.
- 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, dilute the samples with Calibrator Diluent (1×) and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.

III. ADVANTAGES

A. PRECISION

Intra-assay Precision (Precision within an assay)

Three samples were tested twenty times on one plate to assess intra-assay precision.

Inter-assay Precision (Precision between assays)

Three samples were tested in forty separate assays to assess inter-assay precision.

	Intra-assay Precision			**Inter-assay Precision**		
Sample	1	2	3	1	2	3
Mean (pg/mL)	571	1123	2318	518	1073	2159
Standard Deviation	13.2	31.1	83.9	41.2	76.1	123
CV%	2.3	2.8	3.6	8.0	7.1	5.7

B. RECOVERY

The recovery of human Thrombomodulin spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	**Average % Recovery**	**Range (%)**
Cell culture media* (n=4)	96	88-106
Human Serum* (n=4)	89	85-96
Human EDTA plasma* (n=4)	95	86-105
Human Heparin plasma* (n=4)	91	85-96
Human Citrate plasma* (n=4)	94	85-102
Human Urine* (n=4)	99	85-115

*Samples were initially diluted prior to assay as directed in the Sample Preparation section.

C. SENSITIVITY

Forty assays were evaluated and the minimum detectable dose (MDD) of human

Thrombomodulin ranged from 2.91-27.0 pg/mL. The mean MDD was 7.82 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.

D. CALIBRATION

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

E. LINEARITY

To assess the linearity of the assay, different samples were containing or spiked with high concentrations of human Thrombomodulin and diluted with Calibrator Diluent (1×) to produce samples with values within the dynamic range of the assay.

Dilution		**Cell culture media* (n=4)**	**Human Serum* (n=4)**	**Human EDTA plasma* (n=4)**	**Human Heparin plasma* (n=4)**	**Human Citrate plasma* (n=4)**	**Human Urine* (n=4)**
1:2	Average % of Expected	106	98	98	97	99	102
	Range (%)	103-110	93-102	93-101	94-98	98-100	96-112
1:4	Average % of Expected	107	95	100	96	102	107
	Range (%)	102-111	93-98	94-107	93-101	101-102	103-114
1:8	Average % of Expected	111	94	105	97	105	110
	Range (%)	104-115	85-98	93-113	96-99	104-107	103-115
1:16	Average % of Expected	111	88	107	98	106	110
	Range (%)	108-114	85-92	90-113	95-101	102-111	100-119

*Samples were diluted prior to assay as directed in the Sample Preparation section.

F. SAMPLE VALUES

Human Serum/Plasma/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human Thrombomodulin in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (pg/mL)	Range (pg/mL)	Standard Deviation (pg/mL)
Human Serum (n=35)	4032	2866-5318	637
Human EDTA plasma (n=35)	4226	2815-5407	671
Human Heparin plasma (n=35)	4092	2805-5479	645
Human Citrate plasma (n=35)	3511	2353-4541	535
Human Urine (n=11)	15,800	3540-29,100	8340

Cell Culture Supernates/Cell Lysates:

HUVEC human umbilical vein endothelial cells were cultured in EGM-2 until confluent.

MG-63 Human osteosarcoma cells were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate for 4 days.

U-87 MG human glioblastoma/astrocytoma cells were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 1.0 mM sodium pyruvate, and 100 µg/mL streptomycin sulfate until confluent.

Cell Line	Cell culture supernates (pg/mL)	Cell lysates (pg/mg cell lysate)
HUVEC	ND	5022
MG-63	ND	670
U-87 MG	186	817

ND=Non detectable

G. SPECIFICITY

This assay recognizes natural and recombinant human Thrombomodulin. This assay also recognizes Thrombomodulin complexed with Thrombin, Protein C, and Thrombin/Protein C.

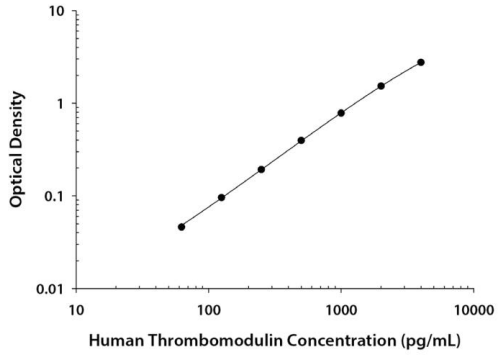
The factors listed below were prepared at 50 ng/mL in Calibrator Diluent (1×) and assayed for cross-reactivity. Preparations of the following factors at 50 ng/mL in a mid-range recombinant human Thrombomodulin control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:	**Recombinant mouse:**	**Natural proteins:**
Coagulation Factor VII	Coagulation Factor VII	human LDL
Coagulation Factor Xa	Coagulation Factor XI	human HDL
Coagulation Factor XI	Thrombomodulin	human VLDL
Protein C		
Protein S		
Thrombin		

IV. EXPERIMENT

EXAMPLE STANDARD

The 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	0.030 0.030	0.030	—
62.5	0.075 0.076	0.076	0.046
125	0.125 0.127	0.126	0.096
250	0.222 0.222	0.222	0.192
500	0.423 0.429	0.426	0.396
1000	0.801 0.822	0.812	0.782
2000	1.555 1.561	1.558	1.528
4000	2.733 2.832	2.783	2.753

V. KIT COMPONENTS AND STORAGE

A. MATERIALS PROVIDED

Parts	Description	Size
Human Thrombomodulin Microplate	96 well polystyrene microplate (12 strips of 8 wells) coated with an antibody against human Thrombomodulin	1 plate
Human Thrombomodulin Conjugate	Solution of antibody against human Thrombomodulin conjugated to horseradish peroxidase	1 vial
Human Thrombomodulin Standard	Recombinant human Thrombomodulin in a buffered protein; lyophilized. Refer to the vial label for reconstitution volume	1 vial
Assay Diluent RD1X	A buffered protein base	1 vial
Calibrator Diluent Concentrate (5×)/ RD5P	A 5× concentrated buffered protein base used to dilute standard and samples	1 vial
Wash Buffer Concentrate (25×)	A 25× concentrated solution of buffered surfactant	1 vial
TMB Substrate	TMB ELISA Substrate Solution/TMB Substrate Solution	2 vials
Stop Solution	2 N sulfuric acid	1 vial
Plate Sealers	Adhesive strip	3 strips

B. STORAGE

<table>
<tr><td>Unopened Kit</td><td colspan="2">Store at 2-8°C. Do not use past kit expiration date.</td></tr>
<tr><td rowspan="6">Opened/
Reconstituted
Reagents</td><td>Wash Buffer (1×)</td><td rowspan="4">May be stored for up to 1 month at 2-8°C.*</td></tr>
<tr><td>Stop Solution</td></tr>
<tr><td>Conjugate</td></tr>
<tr><td>TMB Substrate</td></tr>
<tr><td>Standard</td><td>May be stored for up to 1 month at 2-8 °C.*</td></tr>
<tr><td>Assay Diluent
RD1X</td><td>May be stored for up to 1 month at 2-8 °C.*</td></tr>
<tr><td>Calibrator Diluent
Concentrate (5×)/
RD5P</td><td>May be stored for up to 1 month at 2-8 °C.*
Use and discard diluted Calibrator Diluent (1×). Prepare fresh for each assay.</td></tr>
<tr><td>Microplate Wells</td><td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8°C.*</td></tr>
</table>

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

C. 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.
- If using cell lysate samples, the following is also required:

  • PBS

  • Cell Lysis Buffer 1 (R&D Systems, Catalog # 890713).

D. PRECAUTION

- Some components in this kit contain a preservative which may cause an allergic skin reaction. Avoid breathing mist.
- The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.
- Human Thrombomodulin is detectable in human saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

VI. PREPARATION

A. SAMPLE COLLECTION AND 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 and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Samples may require dilution with Calibrator Diluent (1×).

Cell Lysates - Cells must be lysed prior to assay. Refer to the Cell Lysis Procedure. Samples may require dilution with Calibrator Diluent (1×).

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 × g. Remove serum and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Samples may require dilution with Calibrator Diluent (1×).

Plasma - Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 × g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Samples may require dilution with Calibrator Diluent (1×).

Urine - Aseptically collect the first urine of the day (mid-stream), voided directly into a sterile container. Centrifuge to remove particulate matter, and assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Samples may require dilution with Calibrator Diluent (1×).

B. CELL LYSIS PROCEDURE

Use the following procedure for the preparation of cell lysate samples.

1. Gently wash cells with cold PBS. Pour off and discard the PBS.

2. Add sufficient Cell Lysis Buffer 1 to cover the cells (for example, 10 mL if using a T75 flask) and incubate at room temperature for 30 minutes with gentle agitation.

3. Collect the cell lysates and centrifuge at 12,000 rpm for 10 minutes to remove cell debris.

4. Collect the supernate and determine the total protein concentration of the lysate using the Bradford method (20).

5. Aliquot the lysis supernatant and store at ≤ -80 °C until ready for use.

C. SAMPLE PREPARATION

Cell culture supernate and cell lysate samples recommend a 2-fold dilution. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent (1×). Optimal dilutions should be determined by the end user.

Human serum, plasma, and urine samples recommend a 10-fold dilution. A suggested 10-fold dilution is 20 µL of sample + 180 µL of Calibrator Diluent (1×). Optimal dilutions should be determined by the end user.

D. REAGENT PREPARATION

Bring all reagents to room temperature before use.

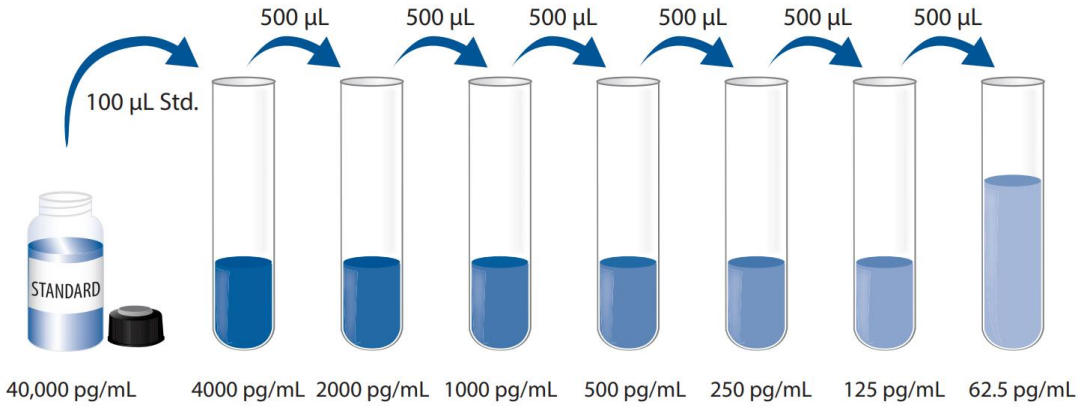
Wash Buffer (1×) - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate (25×) into deionized or distilled water to prepare 500 mL of Wash Buffer (1×).

Calibrator Diluent (1×) - Use deionized or distilled water to prepare Calibrator Diluent (1×).

Human Thrombomodulin Standard - Refer to the vial label for the reconstitution volume*. Reconstitute the Human Thrombomodulin Standard with deionized or distilled water. This reconstitution produces a stock solution of 40000 pg/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

*If you have any question, please seek help from our Technical Support.

Pipette 900 µL of Calibrator Diluent (1×) into the 4000 pg/mL tube. Pipette 500 µL into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 4000 pg/mL standard serves as the high standard. Calibrator Diluent (1×) serves as the zero standard (0 pg/mL).



E. 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.
- It is recommended that the samples be pipetted within 15 minutes.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- TMB Substrate should remain colorless until added to the plate. Keep TMB Substrate protected from light. TMB Substrate should change from colorless to gradations of blue.
- Stop Solution should be added to the plate in the same order as the TMB Substrate. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the TMB Substrate.

VII.ASSAY PROCEDURE

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

1. Prepare all reagents and working standards 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 100 µL of Assay Diluent RD1X to each well. *May contain crystals. Warm to room temperature and mix well to dissolve.*
4. Add 50 µL of standard and prepared sample per well. Cover with the adhesive strip provided. **Incubate for 2 hours at room temperature.** A plate layout is provided for a record of standards and samples assayed.
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 200 µL of Human 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 200 µL of TMB Substrate to each well. **Incubate for 30 minutes at room temperature. Protect from light**.
9. Add 50 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.
10. Determine the optical density of each well within 10 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.

11. CALCULATION OF RESULTS

Average the duplicate readings for each standard 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 human 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.

VIII. REFERENCES

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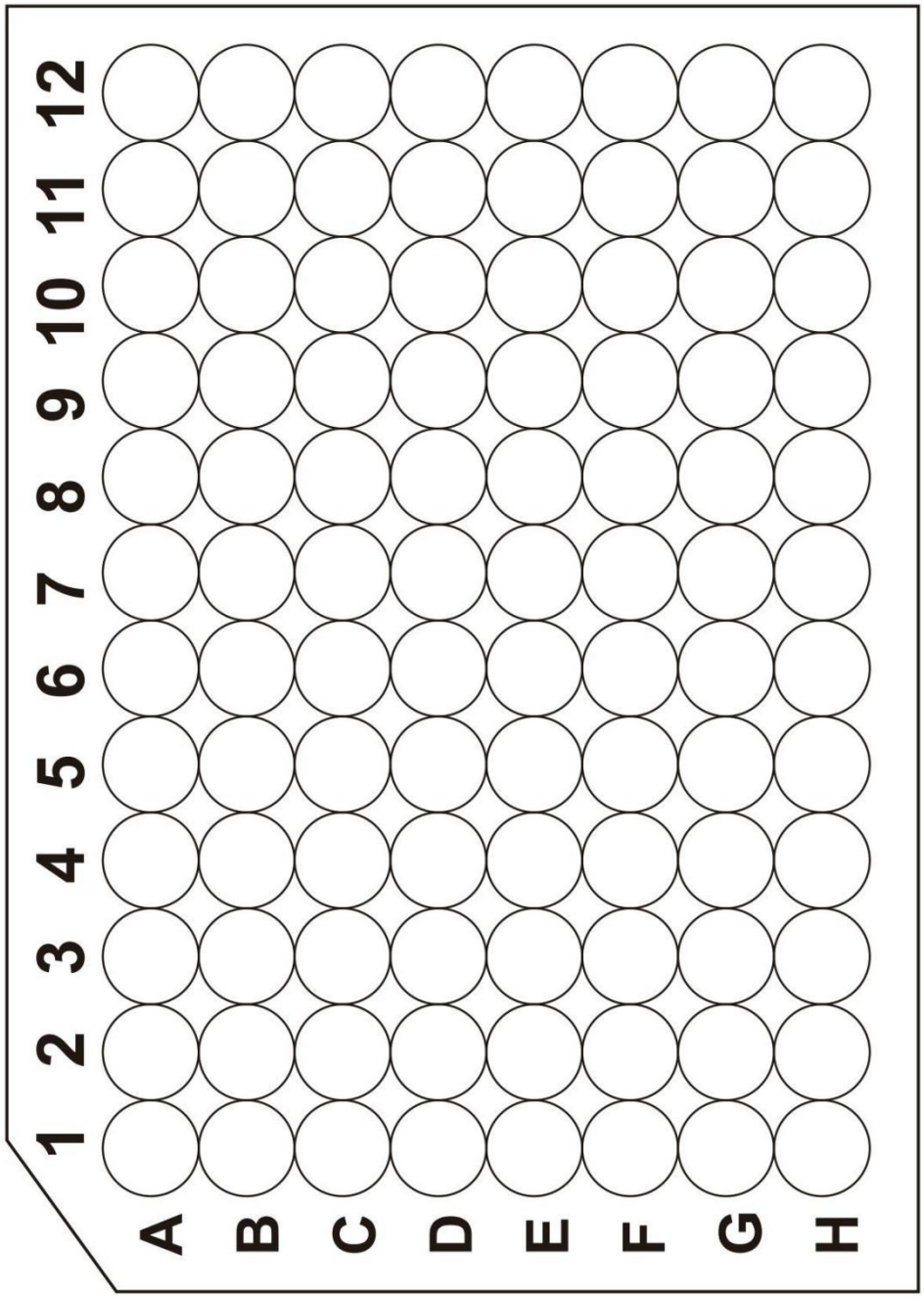
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



产品信息及操作手册

人 Thrombomodulin/BDCA-3 Valukine™ ELISA 试剂盒

目录号：VAL252

适用于定量检测天然和重组人 Thrombomodulin 的浓度

科研专用，不可用于临床诊断

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版本号 202411.1

目录

I. 背景

人类血栓调节蛋白（Thrombomodulin）又称 BDCA-3 和 CD141，是一种跨膜蛋白，主要由血管内皮细胞表达。Thrombomodulin 是抗凝血和纤维蛋白溶解系统的重要组成部分。当凝血级联被激活作用下转化为凝血酶，最终导致纤维蛋白凝块的形成。Thrombomodulin 是凝血酶的细胞表面受体。thrombomodulin-thrombin 复合物可激活蛋白 C 降解凝血因子 Va 和 VIIIa，从而减少凝血酶的生成量并抑制凝血。Thrombomodulin-thrombin 复合物还能激活凝血酶活化性纤维蛋白溶解抑制因子（TAFI），产生一种抑制纤维蛋白溶解的羧肽酶（1-3）。

Thrombomodulin 是一种 I 型跨膜蛋白，由一个 N 端 C 型凝集素结构域、六个连续的表皮生长因子（EGF）样结构域、一个高度糖基化的富丝氨酸/苏氨酸结构域、一个跨膜区段和一个短 C 端胞质尾部组成。类 EGF 结构域对于凝血酶和蛋白 C 的结合至关重要（4）。当血管内皮细胞受到损伤时，thrombomodulin 的胞外结构域会通过蛋白水解作用从细胞表面释放出来，在血液循环中产生多个片段（5, 6）。因此，对血液循环中的可溶性血栓调节蛋白进行定量分析，有助于深入了解血管内皮细胞受损的程度。有资料显示，急性冠状动脉综合征、肺血栓栓塞症和大出血等心血管疾病患者的循环 thrombomodulin 浓度会升高（7-9）。可溶性 thrombomodulin 具有抗凝作用，其治疗潜力已得到探索。初步数据显示，它可能具有预防深静脉血栓形成和治疗弥散性血管内凝血的价值（10-12）。

除抗凝外，thrombomodulin 还有助于其他生物过程。已经证实，蛋白 C 系统以及凝血酶-蛋白酶激活受体系统是重要的凝血因子，在炎症中发挥着重要作用。通过调节这些介质的活性，thrombomodulin 具有抗炎作用（13-14）。Thrombomodulin 还能通过其凝集素结合结构域调节细胞粘附和增殖（15, 16）。此外，由于缺乏功能性 thrombomodulin 的小鼠胚胎无法存活，thrombomodulin 在发育过程中可能具有独特的功能（17, 18）。此外，越来越多的实验证据表明，thrombomodulin 可能能够抑制肿瘤细胞的转移能力，从而在多种恶性肿瘤中发挥保护作用（19）。

II. 概述

A. 检测原理

本实验采用双抗体夹心ELISA法。抗人Thrombomodulin抗体包被于微孔板上，样品和标准品中的人Thrombomodulin会与固定在板上的抗体结合，游离的成分被洗去；加入辣根过氧化酶标记的抗人Thrombomodulin检测抗体进行孵育。洗涤去除未结合的试剂后，加入TMB底物溶液（显色剂）。溶液颜色与结合目标蛋白成正比；加入终止液；用酶标仪测定吸光度。

B. 检测局限

- **仅供科研使用，不可用于体外诊断**。
- 该试剂盒适用于细胞培养上清液、细胞裂解物、人血清、人血浆和人尿液。
- 请在试剂盒有效期内使用。
- 不同试剂盒及不同批号试剂盒的组分不能混用。
- 样本值若大于标准曲线的最高值，应将样本用标准品稀释液（1×）稀释并重复测定。
- 检测结果的不同可由多种因素引起，包括实验人员的操作、移液器的使用方式、洗板技术、反应时间或温度、试剂盒的效期等。

III. 优势

A. 精确度

板内精确度（同一板内不同孔间的精确度）

已知浓度的三个样本，在同一板内分别检测20次，以确定板内精确度。

板间精确度（不同板之间的精确度）

已知浓度的三个样本，在不同板间分别检测40次，以确定板间精确度。

	板内精确度			板间精确度		
样本	1	2	3	1	2	3
平均值（pg/mL）	571	1123	2318	518	1073	2159
标准差	13.2	31.1	83.9	41.2	76.1	123
CV%	2.3	2.8	3.6	8.0	7.1	5.7

B. 回收率

不同类型样本中掺入检测范围内不同水平的人Thrombomodulin，测定其回收率。

样本类型	平均回收率（%）	范围（%）
细胞培养基*（n=4）	96	88-106
人血清*（n=4）	89	85-96
人 EDTA 血浆*（n=4）	95	86-105
人肝素血浆*（n=4）	91	85-96
人柠檬酸盐血浆*（n=4）	94	85-102
人尿液*（n=4）	99	85-115

*样品在分析前按照样品制备部分的指示进行稀释。

C. 灵敏度

评估了 40 次测定，人 Thrombomodulin 的最小可检测剂量（MDD）范围为 2.91-27.0 pg/mL。平均 MDD 为 7.82 pg/mL。

MDD是根据20个重复的零标准品孔的吸光度值的平均值加两倍标准差计算得到的相对应浓度。

D. 校正

该免疫测定法以R&D Systems生产的高纯度的NS0表达的重组人Thrombomodulin校正。

E. 线性

不同的样本中含有或掺入高浓度的人Thrombomodulin，然后用标准品稀释液（1×）将样本稀释到检测范围内，测定其线性。

稀释倍数		细胞培养基*（n=4）	人血清*（n=4）	人EDTA血浆*（n=4）	人肝素血浆*（n=4）	人柠檬酸盐血浆*（n=4）	人尿液*（n=4）
1:2	平均值/期待值（%）	106	98	98	97	99	102
	范围（%）	103-110	93-102	93-101	94-98	98-100	96-112
1:4	平均值/期待值（%）	107	95	100	96	102	107
	范围（%）	102-111	93-98	94-107	93-101	101-102	103-114
1:8	平均值/期待值（%）	111	94	105	97	105	110
	范围（%）	104-115	85-98	93-113	96-99	104-107	103-115
1:16	平均值/期待值（%）	111	88	107	98	106	110
	范围（%）	108-114	85-92	90-113	95-101	102-111	100-119

*样品在分析前按照样品制备部分的指示进行稀释。

F. 样本预值

人血清/血浆/尿液-在本试验中评估了来自表面健康志愿者的样本中是否存在人Thrombomodulin。本研究中使用的供体没有病史。

样本类型	平均值（pg/mL）	范围（pg/mL）	标准偏差（pg/mL）
人血清（n=35）	4032	2866-5318	637
人 EDTA 血浆（n=35）	4226	2815-5407	671
人肝素血浆（n=35）	4092	2805-5479	645
人柠檬酸盐血浆（n=35）	3511	2353-4541	535
人尿液（n=11）	15,800	3540-29,100	8340

细胞培养上清/细胞裂物：

将人脐静脉内皮细胞（HUVEC）在EGM-2培养基中培养至汇合。

将MG-63人骨肉瘤细胞在含有10%胎牛血清、2 mM L-谷氨酰胺、100 U/mL青霉素和100 µg/mL链霉素硫酸盐的DMEM中培养4天。

将U-87 MG人胶质母细胞瘤/星形细胞瘤细胞在含有10%胎牛血清、2 mM L-谷氨酰胺、100 U/mL青霉素、1.0 mM丙酮酸钠和100 µg/mL硫酸链霉素的MEM中培养至汇合。

细胞系	细胞培养上清液（pg/mL）	细胞裂解物（pg/mg cell lysate）
HUVEC	ND	5022
MG-63	ND	670
U-87 MG	186	817

ND=未检出

G. 特异性

该检测方法识别天然和重组人Thrombomodulin。此检测方法还可识别与凝血酶、Protein C以及凝血酶/ Protein C复合的Thrombomodulin。

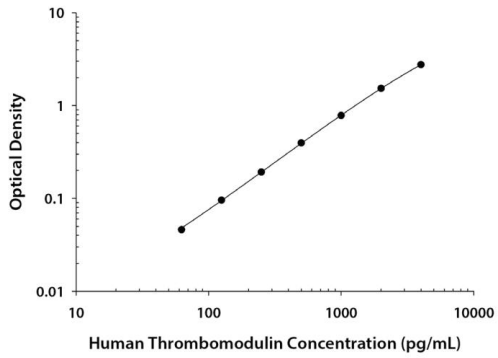
以下列出的因子在标准品稀释液（1×）中以50 ng/mL的浓度制备，并进行交叉反应性测定。以下列出的因子在中值范围重组人Thrombomodulin对照品中以50 ng/mL的浓度制备，并进行干扰测定。未观察到明显的交叉反应或干扰。

Recombinant human:	**Recombinant mouse:**	**Natural proteins:**
Coagulation Factor VII	Coagulation Factor VII	human LDL
Coagulation Factor Xa	Coagulation Factor XI	human HDL
Coagulation Factor XI	Thrombomodulin	human VLDL
Protein C		
Protein S		
Thrombin		

IV. 实验

标准曲线实例

该标准曲线数据仅供参考，每次实验应绘制其对应的标准曲线。



(pg/mL)	O.D.	Average	Corrected
0	0.030 0.030	0.030	—
62.5	0.075 0.076	0.076	0.046
125	0.125 0.127	0.126	0.096
250	0.222 0.222	0.222	0.192
500	0.423 0.429	0.426	0.396
1000	0.801 0.822	0.812	0.782
2000	1.555 1.561	1.558	1.528
4000	2.733 2.832	2.783	2.753

V. 试剂盒组成及储存

A. 试剂盒组成

组成	描述	规格
Human Thrombomodulin Microplate	包被抗人Thrombomodulin抗体的96孔聚苯乙烯板，8孔× 12条	1块板
Human Thrombomodulin Conjugate	酶标检测抗人Thrombomodulin抗体	1瓶
Human Thrombomodulin Standard	重组人Thrombomodulin标准品（冻干），参考瓶身标签进行重溶	1瓶
Assay Diluent RD1X	检测液	1瓶
Calibrator Diluent Concentrate (5×)/ RD5P	浓缩的标准品稀释液（5×），用于稀释标准品和样品	1瓶
Wash Buffer Concentrate (25×)	浓缩洗涤缓冲液（25×）	1瓶
TMB Substrate	TMB ELISA底物溶液/TMB底物溶液	2瓶
Stop Solution	终止液	1瓶
Plate Sealers	封板膜	3张

B. 试剂盒储存

<table>
<tr><td>未开封试剂盒</td><td colspan="2">2-8°C储存；请在试剂盒有效期内使用</td></tr>
<tr><td rowspan="8">已打开，稀释或重溶的试剂</td><td>洗涤液（1×）</td><td rowspan="4">2-8°C储存，最多30天*</td></tr>
<tr><td>终止液</td></tr>
<tr><td>酶标检测抗体</td></tr>
<tr><td>TMB底物溶液</td></tr>
<tr><td>标准品</td><td>2-8°C储存，最多30天*</td></tr>
<tr><td>检测液RD1X</td><td>2-8°C储存，最多30天*</td></tr>
<tr><td>浓缩的标准品稀释液（5×）/ RD5P</td><td>2-8℃储存，最多 30 天*
请每次使用新鲜配制的1×标准品稀释液，多余的丢弃</td></tr>
<tr><td>包被的微孔板条</td><td>将未用的板条放回带有干燥剂的铝箔袋内，密封；
2-8 °C储存，最多30天*</td></tr>
</table>

*必须在试剂盒有效期内

C. 实验所需自备试验器材

- 酶标仪（可测量450 nm检测波长的吸收值及540 nm或570 nm校正波长的吸收值）
- 高精度加液器及一次性吸头
- 蒸馏水或去离子水
- 洗瓶（喷瓶）、多通道洗板器或自动洗板机
- 500 mL量筒
- 用于稀释标准品和样品的管子
- 如果使用细胞裂解物样品，则还需要以下内容：
  - PBS
  - 细胞裂解液1（R&D Systems，货号890713）。

D. 注意事项

- 试剂盒中的一些组分含有防腐剂，可能引起皮肤过敏反应，避免吸入。
- 试剂盒中的终止液是酸性溶液，使用时请做好眼睛、手、面部及衣服的防护。
- 在人唾液中可检测到人Thrombomodulin。在进行此检测时，请采取预防措施以防止试剂盒试剂受到污染。

VI. 实验前准备

A. 样品收集及储存

以下列出的样品收集和储存条件仅作为一般指南。样品稳定性尚未评估。

细胞培养上清液 - 通过离心去除颗粒物，立即或等分进行检测，并将样品储存在≤ -20°C的温度下，避免反复冻融。样品可能需要用标准品稀释液（1×）稀释。

细胞裂解物 - 必须在测定之前裂解细胞。请参考细胞裂解步骤。样品可能需要用标准品稀释液（1×）稀释。

血清 - 使用血清分离管(SST)，让样本在室温下凝固30分钟，然后在1000 × g的离心力下离心15分钟。分离血清并立即进行检测，或分装并储存在≤ -20 °C。避免反复冻融。样品可能需要用标准品稀释液（1×）稀释。

血浆 - 使用肝素、EDTA或柠檬酸作为抗凝剂收集血浆。在采样后30分钟内，以1000 × g的离心力离心15分钟。立即检测或分装并储存在≤ -20 °C。避免反复冻融。样品可能需要用标准品稀释液（1×）稀释。

尿液 - 无菌收集晨尿（中段），直接排入无菌容器。离心去除颗粒物，立即检测或分装并储存于在≤ -20 °C。避免反复冻融。样品可能需要用标准品稀释液（1×）稀释。

B. 细胞裂解

按照以下步骤制备细胞裂解样品。

1. 用冰PBS轻轻冲洗细胞。倒掉并丢弃PBS。
2. 加入足够的细胞裂解液1，使细胞完全浸没（如使用T75培养瓶时，加入10 mL），并在室温下轻轻晃动孵育30分钟。
3. 收集细胞裂解液，并以12,000 rpm 离心10分钟，去除细胞碎片。
4. 收集上清液，并用Bradford法测定裂解液的总蛋白浓度（20）。
5. 取裂解上清液，并储存于≤ -80 °C，待使用时再取出。

C. 样品准备

细胞培养上清液和细胞裂解物样本建议稀释2倍。推荐的2倍稀释方法为100 µL样本+100 µL标准品稀释液（1×）。最佳稀释倍数应由用户确定。

人血清、血浆和尿液样本建议稀释 10 倍。推荐的 10 倍稀释方法为 20 µL 样本+180 µL 标准品稀释液（1×）。最佳稀释倍数应由用户确定。

D. 检测前准备工作

使用前请将所有试剂放置于室温。

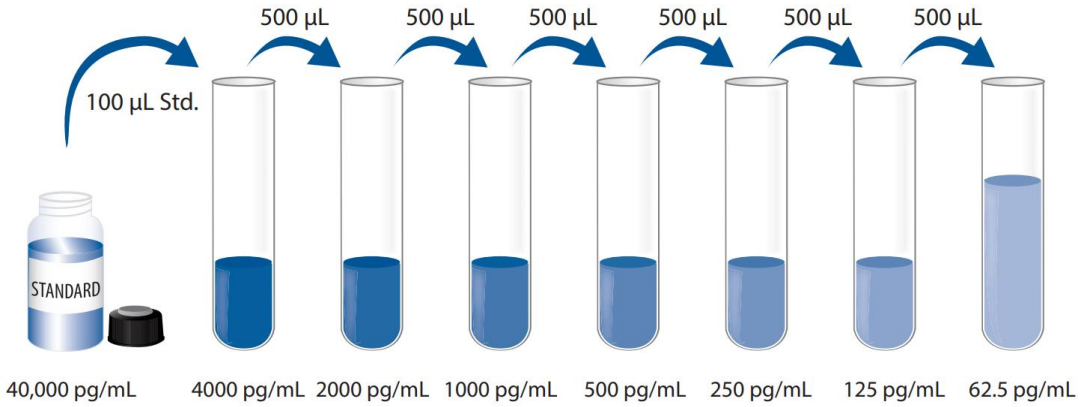
洗涤液（1×）：从冰箱中取出的浓缩洗涤液可能有结晶，属于正常现象；放置室温，轻摇混匀，待结晶完全溶解后再配制洗涤液。可将20 mL浓缩洗涤液（25×）用蒸馏水或去离子水稀释配制成500 mL工作浓度的洗涤液（1×）。

标准品稀释液（1×）：使用去离子水或蒸馏水制备标准品稀释液（1×）。

人Thrombomodulin标准品：重溶体积请参考瓶身标签*，用去离子水或蒸馏水重溶人Thrombomodulin标准品，得到浓度为40000 pg/mL标准品储备母液。轻轻震摇至少15分钟，其充分溶解。

*如有疑问，请咨询我们的技术支持。

用移液管将900 µL标准品稀释液（1×）移入4000 pg/mL的管中。其余管中均加入500 µL准品稀释液（1×）。使用标准品储备母液稀释（如下）。在每次转移之前，将每个管彻底混合。4000 pg/mL作为标准曲线最高点。标准品稀释液（1×）作为标准曲线零点（0 pg/mL）。



E. 技术小提示

- 当混合或重溶蛋白液时，尽量避免起沫。
- 为了避免交叉污染，配制不同浓度标准品、上样、加不同试剂都需要更换枪头。另外不同试剂请分别使用不同的移液槽。
- 建议15分钟内完成一块板的上样。
- 每次孵育时，正确使用封板膜可保证结果的准确性。
- TMB底物溶液在上板前应为无色，请避光保存；加入微孔板后，将由无色变成不同深度的蓝色。
- 终止液上板顺序应同TMB底物溶液上板顺序一致；加入终止液后，孔内颜色由蓝变黄； 若孔内有绿色，则表明孔内液体未混匀请充分混合。

VII.操作步骤

使用前，将所有其他试剂和样品放置于室温。建议对所有标准品和样品进行复孔检测。

1. 按照上一节的说明，准备好所有需要的试剂和标准品。
2. 从已平衡至室温的密封袋中取出微孔板，未用的板条请放回铝箔袋内，重新封口。
3. 向每个孔中加入100 µL的检测液RD1X。*可能含有结晶，使其回温至室温并充分混匀至溶解。*
4. 分别将不同浓度标准品和实验样本加入相应孔中，每孔50 µL。用封板膜封住反应孔，**室温孵育2小时**。说明书提供了一张96孔模板图，可用于记录标准品和试验样本的板内位置。
5. 将板内液体吸去，使用洗瓶、多通道洗板器或自动洗板机洗板。每孔加洗涤液400 µL，然后将板内洗涤液吸去。重复操作3次，共洗4次。每次洗板尽量吸去残留液体会有助于得到好的实验结果。最后一次洗板结束，请将板内所有液体吸干或将板倒置，在吸水纸拍干所有残留液体。
6. 在每个微孔内加入200 µL人Thrombomodulin酶标检测抗体。用封板膜封住反应孔，**室温孵育2小时**。
7. 重复第5步洗板操作；
8. 在每个微孔内加入200 µL TMB底物溶液，**室温孵育30分钟。注意避光**。
9. 在每个微孔内加入50 µL终止液，请轻拍微孔板，使溶液混合均匀。
10. 加入终止液后10分钟内，使用酶标仪测量450 nm的吸光度值，设定540 nm或570 nm作为校正波长。如果波长校正不可用，以450 nm的读数减去540 nm或570 nm的读数。这种减法将校正酶标板上的光学缺陷。没有校正而直接在450 nm处进行的读数可能会更高且更不准确。
11. 计算结果：

    将每个标准品和样品的复孔吸光值取平均值，然后减去零标准品平均OD值（O.D.），使用计算机软件作四参数逻辑（4-PL）曲线拟合创建标准曲线。另一替代方法是，通过绘制y轴上每个标准品的平均吸光值与x轴上的浓度来构建标准曲线，并通过图上的点绘制最佳拟合曲线。数据可以通过绘制人Thrombomodulin浓度的对数与O.D.的对数来线性化，并且最佳拟合线可以通过回归分析来确定。该程序将产生足够但不太精确的数据拟合。

    如果样品被稀释，从标准曲线读取的浓度必须乘以稀释倍数。

VIII. 参考文献

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96 孔模板图

请使用 96 孔模板图来记录标准品及样本在板内的位置