Nori® Rat Thrombomodulin ELISA Kit Datasheet

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.[1] Thrombomodulin is encoded by the THBD gene.[2] The protein has a molecular mass of 74kDa, and consists of a single chain with six tandemly repeated EGF-like domains, a Serine/Threonine-rich spacer and a transmembrane domain.[1] Thrombomodulin functions as a cofactor in the thrombin-induced 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 thousand-fold. 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) have also been reported to be associated with atypical hemolytic-uremic syndrome (aHUS).Thrombomodulin is also expressed on human mesothelial cell,[3] monocyte and a dendritic cell subset. The antigen described as BDCA-3[4] has turned out to be identical to thrombomodulin.[5] Thus, it was revealed that this molecule also occurs on a very rare (0.02%) subset of human dendritic cells called MDC2. Its function on these cells is unknown.

References

1. Sadler JE (1997). Thrombosis and haemostasis. 78 (1): 392–5.
2. Wen DZ, et al. (1987). Biochemistry. 26 (14): 4350–7.
3. Verhagen HJ, et al. (1996). British Journal of Haematology. 95 (3): 542–9.
4. Dzionek A, et al. (2000). Journal of Immunology. 165 (11): 6037–46.
5. Dzionek A, et al. (2002). Human Immunology. 63 (12): 1133–48.

PRINCIPLE OF THE ASSAY

This ELISA kit is for quantification of Thrombomodulin. This is a shorter ELISA assay that reduces time to 50% compared to the conventional method, and the entire assay only takes 3 hours. This assay employs the quantitative sandwich enzyme immunoassay technique and uses biotin-streptavidin chemistry to improve the performance and the sensitivity of the assays. An antibody specific for Thrombomodulin has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Thrombomodulin present is bound by the immobilized antibody. After washing away any unbound substances, a detection antibody specific for Thrombomodulin is added to the wells. Following wash to remove any unbound antibody reagent, a detection reagent is added. After intensive wash a substrate solution is added to the wells and color develops in proportion to the amount of Thrombomodulin bound in the initial step. The color development is stopped, and the intensity of the color is measured.

Please ensure the kit contents such as HRP volume when received and this package insert must be read in its entirety before using this product.

Storage

Store the kit at 4°C. The kit can be used in 6 months.

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MATERIALS PROVIDED

Description	Quantity	Description	Quantity	Description	Quantity
Antibody Precoated Plate	1	20 x PBS	1	Substrate Solution	1
Detection Antibody	1	20 x Assay Buffer	1	Stop Solution	1
HRP Conjugate	1	96-well plate sheet	1	Datasheet	1
Standard	3				

Bring all reagents to room temperature before use.

Reagent Preparations

Rat Thrombomodulin Detection Antibody (1 vial) – The lyophilized Detection Antibody should be stored at 4°C for up to 6 months, if not used immediately. Centrifuge for 1 min at 6000 x g to bring down the material prior to open the vial. The vial contains sufficient Detection Antibody for a 96-well plate. Add 200 µL of sterile 1 x PBS and vortex 30 sec. If the entire 96-well plate is used, take 200 µL of detection antibody to 10.5 mL of 1 x PBS to make **Working dilution of detection antibody** and mix thoroughly prior to the assay. If the partial antibody is used store the rest at -20°C until use.

Rat Thrombomodulin Standard (3 vials) – Each lyophilized Standard vial contains the standard sufficient for generating a standard curve. The unreconstituted standard can be stored at 4°C for up to 6 months if not used immediately. Centrifuge for 1 min at 6000 x g to bring down the material prior to open the vial. Add 500 µL of 1 x Assay Buffer to make the high standard concentration of 4000 pg/mL and **vortex for 30 sec.** Centrifuge at 3000 x g for 1 min to bring down all the materials and remove insoluble if any. A seven-point standard curve is generated using 2-fold serial dilutions in the Assay Buffer, **vortex 10 sec for each of dilution step**.

HRP Conjugate (55 µL) – Centrifuge for 1 min at 3000 x g to bring down the material prior to open the vial. The vial contains 55 µL HRP Conjugate sufficient for one 96-well plate (Please notify us if it is below 40 µL). Add 1 x PBS to reach 55 µL if needed. Make 1:200 dilutions in 1 x PBS. If the entire 96-well plate is used, add all HRP Conjugate to 10.5 mL of 1 x PBS to make **working dilution of HRP Conjugate** and mix thoroughly prior to the assay. The rest of undiluted HRP Conjugate can be stored at 4 °C for up to 6 months. It is recommended to fully recover the HRP Conjugate with 1 mL of 1 x PBS at final use.

20 x PBS, pH 7.3, 25 mL- Dilute to 1 x PBS with deionized distilled water and mix well prior to use.
20 x Assay Buffer, 20 mL- Dilute to 1 x Assay Buffer with 1 x PBS prior to use.
Substrate Solution, 10.5 mL.
Stop Solution, 5.5 mL.

Sample Types: Plasma, serum, cell/tissue lysates, cell culture, synovial fluid (SF), bronchoalveolar lavage (BAL), cerebrospinal fluid (CSF), urine, and other biological fluid.

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Assay Procedure

1. All procedures are conducted at room temperature (20-25 ºC) and ensure **equal pipetting/dispensing** at each step and remove air bubbles in the wells for all steps.
2. Lift the plate cover and cover the unused wells or reseal the unused strips in the aluminum bag with desiccant at 4 ºC. **Vortex the standards and samples for 10 sec** before applying to the plate. Add 100 µL of **sample** or **standard** per well and use duplicate wells for each standard or sample. Cover the 96-well plate and incubate on shaker at 450 rpm for **1 h** (1.5 h if no shaking). Attention: **MUST vortex standards and samples for 10 sec before pipetting to the wells!**
3. Aspirate each well and wash with 300 µL of **1 x Assay Buffer** for two times. Wash by filling each well with 1 x Assay Buffer using a multi-channel pipette, manifold dispenser, squirt bottle or auto-washer. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Assay Buffer by aspirating or by inverting the plate and blotting it against clean paper towels.
4. Add 100 µL of the **working dilution of Detection Antibody** to each well. Cover the plate and incubate on shaker at 450rpm for 1 h (1.5 h if no shaking).
5. Repeat the aspiration/wash as in step 3.
6. Add 100 µL of the **working dilution of HRP Conjugate** to each well. Cover the plate and incubate for 20 minutes. Avoid placing the plate in direct light.
7. Repeat the aspiration/wash as in step 3 but **wash 4 times** instead.
8. Add 100 µL of **Substrate Solution** to each well and observe the color development every 1-2 mins. Incubate for up to **30 minutes depending on signal (Stop** the reaction when it turns to dark blue in the highest standard well). Over-incubation of the substrate will result in overflow of high standard and should be avoided. Avoid placing the plate in direct light.
9. When it gets to dark blue in the highest concentration of standard well, add 50 µL of **Stop Solution** to each well to stop the reaction. Gently tap the plate to ensure thorough mixing. **Ensure all wells turn yellow by pipette tip prior to measurement.**
10. Determine the optical density of each well immediately, using a validated 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.

Sample dilution: If high density is expected, samples should be diluted with equal volume of 1 x Assay Buffer and **vortex for 30 sec** prior to the assay. If the OD value still exceeds the upper limit of the standard curve, further dilution is recommended till it falls in the detection range and the dilution factor must be used for calculation of the concentration.

Precaution and Technical Notes

1. It is critical to follow the procedure step by step otherwise appropriate color development may not occur as expected and make sure no air bubbles in wells before adding reagents.
2. A standard curve should be generated for each set of samples assayed. Thorough mixing of standards at each of dilution steps is critical to acquire a normal standard curve and **vortex again (10 sec) before pipetting to the 96-well plate**.
3. HRP Conjugate contains enzyme, DO NOT mass up with Detection Antibody.
4. The Stop Solution is an acid solution, handle with caution.
5. This kit should not be used beyond the expiration date on the label.
6. A thorough and consistent wash technique is essential for proper assay performance.
7. Use a fresh reagent reservoir and pipette tips for each step.
8. It is recommended that all standards and samples be assayed in duplicate.
9. Avoid microbial contamination of reagents and buffers. This may interfere with the performance of the assay.

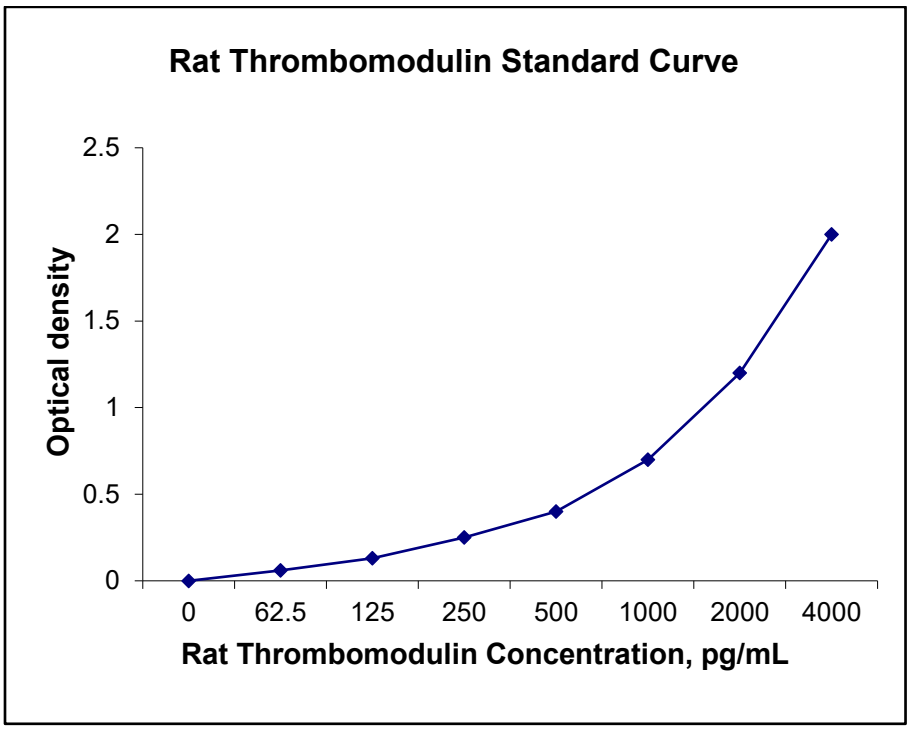
Calculation of Results

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

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 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.

The Standard Curve

The graph below represents typical data generated when using the ELISA Kit. The correlation coefficient (r^2) is 0.995-1.000. The standard curve should be calculated using a computer generated 4-PL curve-fit to determine concentrations of unknow specimens.



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Specificity

The following recombinant Rat proteins prepared at 5 ng/mL were tested and exhibited no cross-reactivity or interference.

ApoAI, BMP7, CRP, HGF, HSP27, IL-1α, IFNγ, MMP-2, prolactin, TGFβ1, TLR3, TNF-α, VEGF.

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Detection Range

62.5-4,000 pg/mL

Assay Sensitivity

12 pg/mL

Assay Precision

Intra-Assay %CV: 6; Inter-Assay %CV: 9

Assay Accuracy

Intra-Assay: 87-113%; Inter-Assay: 85-114%

Related products

1. GR238016 50 ml Reagent Reservoir, 100/pack, 5 bags/pack (20/bag)
2. GR238031 96-well microplate sealer plastic, colorless, pack of 100
3. GR238019 1.5 ml Microcentrifuge tube with screw cap and free-standing, pack of 500
4. GR238004 Tissue Culture 96-well Microplate, individually packed, Case of 50
5. GR238007 125 ml leak-resistant HDPE bottle, colorless, pack of 24
6. GR238002 Microplate 12x8-Well Strip High Binding, colorless, Case of 50
7. GR238003 Microplate 12x8-Well Strip Medium Binding, colorless, Case of 50
8. GR238032 42592 Costar Stripwell Microplate 1 x 8 Flat Bottom, High Binding, Case of 100
9. GR238001 468667 Thermo Microplate 12x8-Well Strip Nunc Maxixorp F8, Case of 60
10. GR238026 Polyethylene Bubble Mailer (Envelope) Self-Sealing 9.1 x 7.1 in, Pack of 50
11. GR238025 Polyethylene Bubble Mailer (Envelope) Self-Sealing 12.2 x 8.1 in, Pack of 25
12. GR238027 Polyethylene Bubble Mailer (Envelope) Self-Sealing 11.8 x 9.8 in, Pack of 20

DECLARATION

THIS REAGENT IS FOR IN VITRO LABORATORY TESTING AND RESEARCH USE ONLY. DO NOT USE IT FOR CLINICAL DIAGNOSTICS. DO NOT USE OR INJECT IT IN HUMANS AND ANIMALS.

FOR LABORATORY RESEARCH USE ONLY
NOT FOR USE IN HUMANS AND ANIMALS

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Troubleshooting Guide

Issues	Possible causes	Solutions
Poor standard curve	• Inaccurate pipetting • Insufficient vortexing • OD_{450} too high for the high standard point • Air bubbles in wells. • Standard defect or not fully recovered	• Check pipette calibration and ensure equal dispensing. • Vortex 30 sec for each of standard dilution steps and vortex again (10 sec) before pipetting to the 96-well plate. • Reduce substrate incubation time • Remove air bubbles in wells by pipette tip. • Change a standard vial or spin down the vial before reconstitution. Centrifuge to remove insoluble if any.
Low signal	• Improper preparation of reagents and storage • Too brief incubation times • Inadequate reagent volume or improper dilution • Standard defect and sample overdiluted	• Spin down vials before opening. Reconstitute the content thoroughly. Proper storage of plate and strip and detection antibody after first usage. • Microplate shaker may improve signals. • Insufficient HRP Conjugate. Ensure sufficient incubation time and increase sample incubation to 2 h. • Change a Standard vial. Undilute sample or less dilution
Overflow in the standards	• Substrate incubation too long • Air bubbles in wells	• Observe the color development every 1-2 mins and reduce substrate incubation time. • Stop the reaction by adding 50 µl of Stop Solution when it turns to dark blue in the highest concentration of standard wells. • Remove air bubbles in wells
Large CV	• Inaccurate pipetting and mixing • Improper standard/sample dilutions. • Air bubbles in wells. • Microplate reader out of calibration • It did not turn yellow after adding Stop Solution	• Check pipettes and ensure the pipette is calibrated properly. • Ensure accurate pipetting and thorough mixing. • Use reverse, instead of forward pipetting. • Use the correct dilution buffers • Remove air bubbles in wells by pipette tip. • Calibrate the microplate reader properly • If it did not turn yellow after adding Stop Solution, mix with pipette tip till it turns yellow prior to measurement.
High background	• Reagent reservoir issue • Plate is insufficiently washed and air bubbles in wells. • Contaminated Assay Buffer • Pipet tip contaminated	• Use a new reagent reservoir for Substrate Solution. • Increase wash to 4 times before adding substrate and ensure plate washer functions normally. Remove air bubbles in wells by pipette tip. Use squirt bottle for washing. • Make fresh Assay Buffer and wash thoroughly. • Use new pipette tips for blank wells.
No signal detected	• The procedure was misconducted. • Failures of spin down the contents in Detection Antibody and Standards. • Failure of HRP or Substrate Samples overdiluted	• Ensure the step-by-step protocol. Spin vials of Detection antibody and Standard to completely recover the content. • Ensure HRP volume. Mix 100 µl of Substrate with 0.5 µl HRP and dark blue color should develop in 5 min. • Try a new standard vial and use positive control. • Try not dilute samples
Low sensitivity	• Improper dilutions of standards • Improper storage of the ELISA kit	• Ensure accurate and thorough dilutions of standards at each step. • Store detection antibody at -20°C after reconstitution and others at 4°C. Keep substrate solution protected from light.