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R&D SYSTEMS

Quantikine® ELISA

Human Thrombomodulin/BDCA-3 Immunoassay

Catalog Number DTHBD0

For the quantitative determination of human Thrombomodulin concentrations in cell culture supernates, cell lysates, 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

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).

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

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human 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, an enzyme-linked monoclonal antibody specific for human Thrombomodulin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, 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.

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 the samples with calibrator diluent and repeat the assay.
- Any variation in 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.
- When using an automated plate washer, adding a 30 second soak period following the addition of Wash Buffer, and/or rotating the plate 180 degrees between wash steps may improve assay precision.
- 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. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate 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
Human Thrombomodulin Microplate	893708	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human 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.*
Human Thrombomodulin Conjugate	893709	21 mL of a monoclonal antibody specific for human Thrombomodulin conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Thrombomodulin Standard	893710	Recombinant human Thrombomodulin in a buffered protein solution with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1X	895121	11 mL of a buffered protein base with preservatives. *May contain crystals. Warm to room temperature and mix well to dissolve.*	
Calibrator Diluent RD5P	895151	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:5 in this assay.*	
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	895032	6 mL of 2 N sulfuric 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.
- Human Thrombomodulin Controls (optional; R&D Systems®, Catalog # QC169).

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

- PBS
- Cell Lysis Buffer 1 (R&D Systems®, Catalog # 890713).

PRECAUTIONS

Thrombomodulin is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

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

Some components in this kit contain a preservative 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. 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 and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Cell Lysates - Cells must be lysed prior to assay. Refer to the Cell Lysis Procedure.

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 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, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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.

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.

SAMPLE PREPARATION

Cell culture supernate and cell lysate samples require a 2-fold dilution. A suggested 2-fold dilution is 100 µL of sample + 100 µL of Calibrator Diluent RD5P (diluted 1:5)*.

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 RD5P (diluted 1:5)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

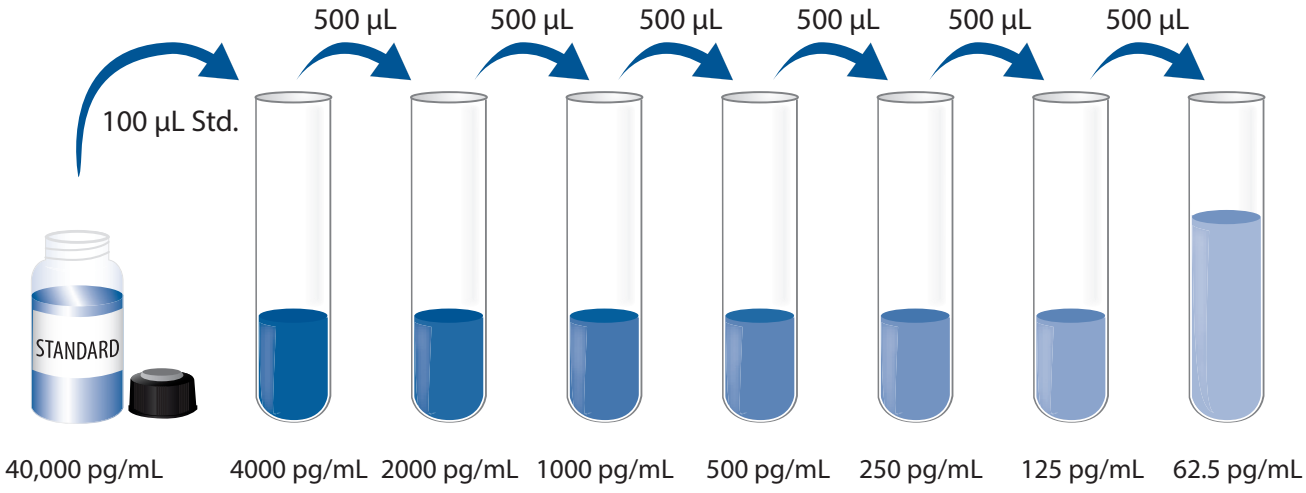
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5P (diluted 1:5) - Add 10 mL of Calibrator Diluent RD5P to 40 mL of deionized or distilled water to prepare 50 mL of Calibrator Diluent RD5P (diluted 1:5).

Human Thrombomodulin Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Thrombomodulin Standard with deionized or distilled water. This reconstitution produces a stock solution of 40,000 pg/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5P (diluted 1:5) 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 RD5P (diluted 1:5) 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, samples, and controls be assayed in duplicate.

1. Prepare all reagents, working standards, 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 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, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. A plate layout is provided to record samples and standards 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 Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, 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 require lysis and/or dilution. See Sample Preparation section.

CALCULATION OF RESULTS

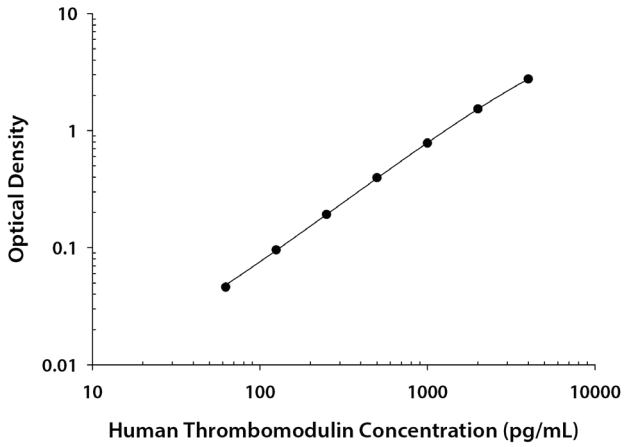
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 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.

Since 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	0.030 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

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 forty separate assays to assess inter-assay precision. Assays were performed by at least three technicians using two lots of components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
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

RECOVERY

The recovery of human Thrombomodulin spiked to levels throughout the range of the assay in various matrices was evaluated. Samples were diluted prior to assay as directed in the Sample Preparation section.

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

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of human Thrombomodulin were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay as directed in the Sample Preparation section.

		Cell culture media (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Citrate plasma (n=4)	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

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 O.D. value of twenty zero standard replicates and calculating the corresponding concentration.

CALIBRATION

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

SAMPLE VALUES

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)
Serum (n=35)	4032	2866-5318	637
EDTA plasma (n=35)	4226	2815-5407	671
Heparin plasma (n=35)	4092	2805-5479	645
Citrate plasma (n=35)	3511	2353-4541	535
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

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 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:

Coagulation Factor VII
Coagulation Factor Xa
Coagulation Factor XI
Protein C
Protein S
Thrombin

Recombinant mouse:

Coagulation Factor VII
Coagulation Factor XI
Thrombomodulin

Natural proteins:

human LDL
human HDL
human VLDL

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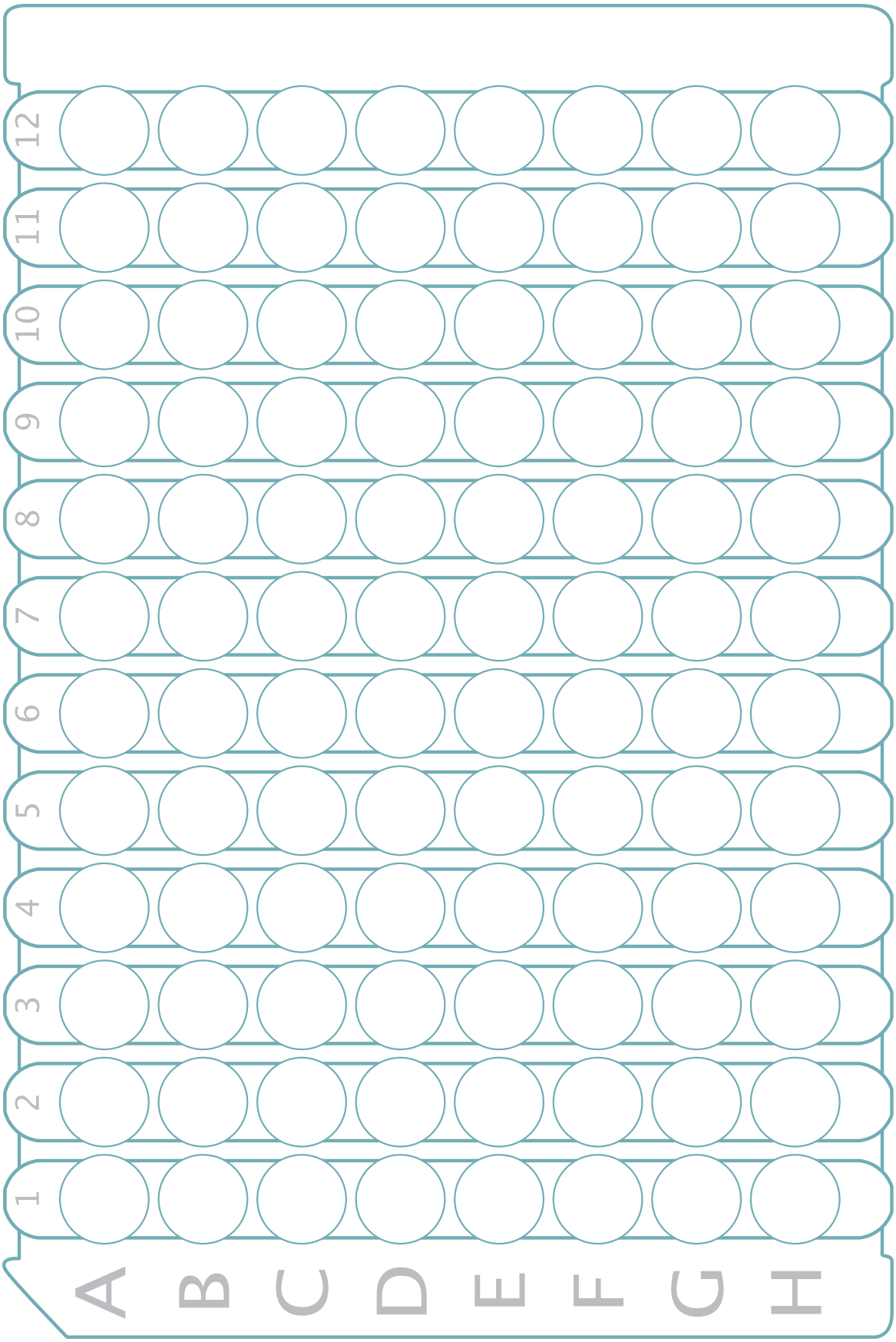
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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