Quantikine® ELISA

Human Thrombospondin-2 Immunoassay

Catalog Number DTSP20

For the quantitative determination of human Thrombospondin-2 concentrations in cell culture supernates, serum, plasma, and human milk.

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

Thrombospondin-2 (also known as TSP-2 and THBS-2) is a 150 kDa calcium-binding protein and member of the Thrombospondin family of secreted proteins known to affect multiple cellular activities including proliferation, aggregation, motility, angiogenesis, tumor progression, and wound healing (1-4). Thrombospondin-2 is known to modulate cellular interactions with extracellular matrix, and as such, is considered a matricellular protein. Thrombospondin-1 and -2 constitute subgroup A within the Thrombospondin family and form disulfide-linked homotrimers. In contrast, Thrombospondin-3, -4, and -5/COMP (cartilage oligomeric matrix protein) constitute subgroup B and form homopentamers (3, 5-7). The human Thrombospondin-2 cDNA encodes a 1172 amino acid (aa) precursor that includes an 18 aa signal sequence followed by an N-terminal heparin-binding domain, an oligomerization motif, one vWF-C (von Willebrand factor type C) domain, three Thrombospondin type-1 repeats, three EGF-like repeats, seven Thrombospondin type-3 repeats, and a lectin-like Thrombospondin C-terminal globular domain (8). Human Thrombospondin-2 shares 88-90% aa sequence identity with bovine, mouse, and rat Thrombospondin-2. Within the Thrombospondin type-3 repeats and Thrombospondin C-terminal domain, human Thrombospondin-2 shares 80% aa sequence identity with human Thrombospondin-1 and approximately 60% aa sequence identity with human Thrombospondin-3, -4, and -5/COMP.

The many reported activities of Thrombospondin-2 are mediated by binding to an array of putative receptors. The heparin-binding domain mediates interactions with several integrins including $\alpha_3\beta_1$ and $\alpha_6\beta_1$ on microvascular endothelial cells (EC), and integrin $\alpha_4\beta_1$ on large blood vessel EC (9, 10). Trimerization of Thrombospondin-2 is required for calcium-dependent cell attachment and spreading, while the heparin-binding domain is shown to mediate destabilization of focal adhesions (11-13). Thrombospondin-2 may also interact directly with integrins via an RGD (Arg-Gly-Asp) sequence located in the type-3 repeat region, or indirectly through C-terminal-binding to integrin-associated protein (IAP/CD47) (14). A fragment of Thrombospondin-2 (heparin-binding domain, oligomerization motif, and vWF-C domain) promotes EC survival, proliferation, and chemotaxis (9). In general, Thrombospondin-2 counteracts blood vessel growth. The three Thrombospondin type-1 domains inhibit VEGF-induced EC migration and vascular tube formation (15, 16). *In vivo*, full length Thrombospondin-2 can block tumor angiogenesis and induce vascular EC apoptosis (15, 17). Histidine-proline-rich glycoprotein (HPRG) functions as an apparent decoy by preventing interaction of Thrombospondin-2 with another putative receptor, CD36, on macrophages and vascular EC (16). Thrombospondin-2 also binds matrix metalloproteinase 2 (MMP-2) and facilitates MMP-2 clearance by the scavenger receptor LRP (LDL receptor-related protein) (18). Thrombospondin-2 regulates collagen matrix formation by altering fibroblast behavior during development and in areas of tissue remodeling in the adult (19, 20). In the heart, Thrombospondin-2 may also have roles in maintaining myocardial matrix integrity, and in the nervous system, it has been shown to promote synaptogenesis (21, 22).

The Quantikine® Human Thrombospondin-2 Immunoassay is a 4.5 hour solid-phase ELISA designed to measure human Thrombospondin-2 in cell culture supernates, serum, plasma, and human milk. It contains NS0-expressed recombinant human Thrombospondin-2 and has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Thrombospondin-2 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 Thrombospondin-2.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Thrombospondin-2 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Thrombospondin-2 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Thrombospondin-2 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 Thrombospondin-2 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 the appropriate 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.
- 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 Thrombospondin-2 Microplate	893164	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Thrombospondin-2.	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 Thrombospondin-2 Conjugate	893165	21 mL of a polyclonal antibody specific for human Thrombospondin-2 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Thrombospondin-2 Standard	893166	Recombinant human Thrombospondin-2 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-75	895811	11 mL of a buffered protein base with preservatives. *May contain a precipitate. Mix well before and during use.*	
Calibrator Diluent RD5P Concentrate	895151	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:3 for serum, plasma and human milk samples. Use diluted 1:10 for cell culture supernate 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.
- Horizontal orbital microplate shaker (0.12" orbit) capable of maintaining a speed of 500 ± 50 rpm.
- **Polypropylene** test tubes for dilution of standards and samples.
- Human Thrombospondin-2 Controls (optional; R&D Systems®, Catalog # QC171).

PRECAUTIONS

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.

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 or heparin 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.

Note: *Citrate plasma has not been validated for use in this assay.*
Hemolyzed samples are not suitable for use in this assay.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and repeat this process a total of 3 times. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Use polyropylene tubes.

Serum and plasma samples require a 4-fold dilution. A suggested 4-fold dilution is 50 µL of sample + 150 µL of Calibrator Diluent RD5P (diluted1:3)*.

* 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.

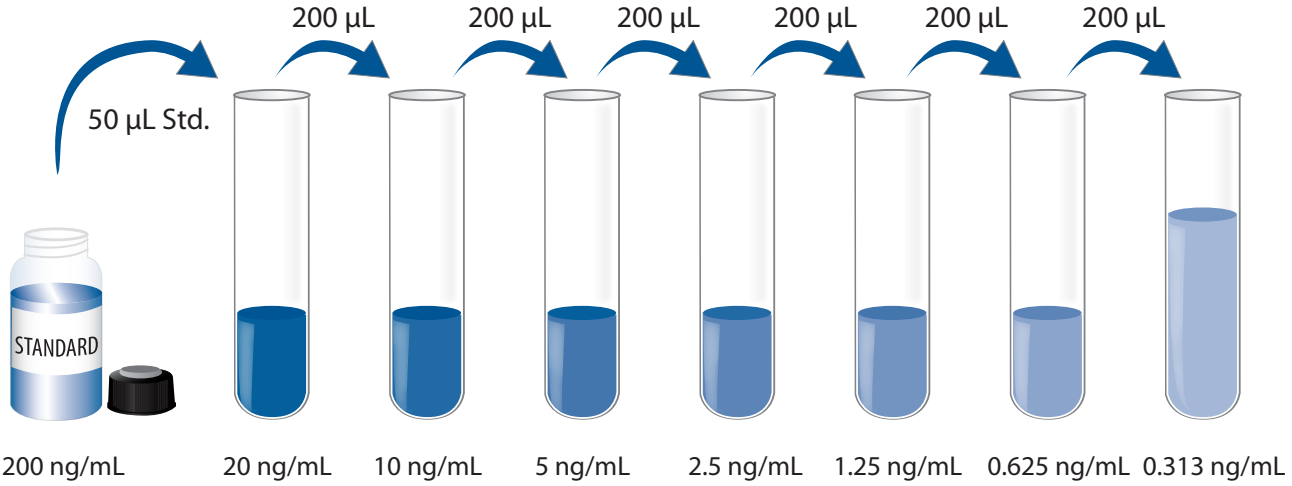
Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 µL of the resultant mixture is required per well.

Calibrator Diluent RD5P (diluted 1:3) - **For serum, plasma, and human milk samples**. Add 5.0 mL of Calibrator Diluent RD5P Concentrate into 10 mL of deionized or distilled water to prepare 15 mL of Calibrator Diluent RD5P (diluted 1:3).

Calibrator Diluent RD5P (diluted 1:10) - **For cell culture supernate samples.** Add 1.0 mL of Calibrator Diluent RD5P Concentrate into 9.0 mL of deionized or distilled water to prepare 10 mL of Calibrator Diluent RD5P (diluted 1:10).

Human Thrombospondin-2 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Thrombospondin-2 Standard with deionized or distilled water. This reconstitution produces a stock solution of 200 ng/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.

Use polypropylene tubes. Pipette 450 µL of Calibrator Diluent RD5P (diluted 1:3) (*for serum, plasma, and human milk samples*) or Calibrator Diluent RD5P (diluted 1:10) (*for cell culture supernate samples*) into the 20 ng/mL tube. Pipette 200 µL of the appropriate calibrator diluent into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 20 ng/mL standard serves as the high standard. The appropriate calibrator diluent serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards 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 RD1-75 to each well. *Assay Diluent RD1-75 may contain a precipitate. Mix well before and during use.*
4. Add 50 µL of standard, control, or sample* per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. A plate layout is provided to record 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 Thrombospondin-2 Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.
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 **on the benchtop. 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 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.).

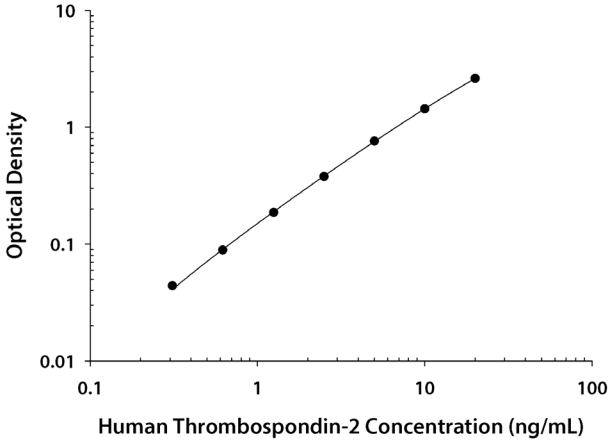
Create a standard curve by reducing the data using computer software capable of generating a four parameter logistic (4-PL) curve-fit. As an alternative, construct a standard curve by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draw a best fit curve through the points on the graph. The data may be linearized by plotting the log of the human Thrombospondin-2 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

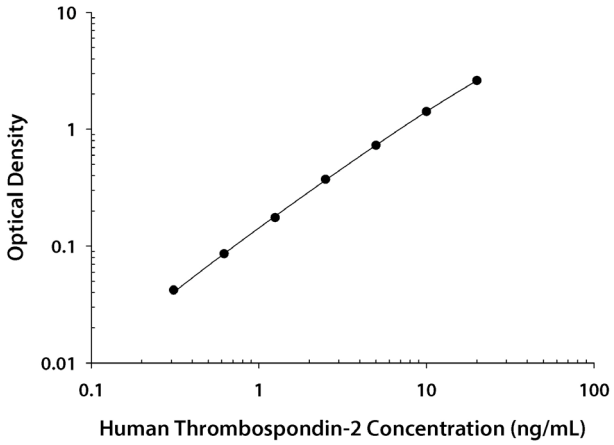
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

CELL CULTURE SUPERNATE ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.023 0.024	0.024	—
0.313	0.067 0.069	0.068	0.044
0.625	0.111 0.115	0.113	0.089
1.25	0.210 0.212	0.211	0.187
2.5	0.402 0.404	0.403	0.379
5	0.781 0.788	0.785	0.761
10	1.455 1.463	1.459	1.435
20	2.630 2.638	2.634	2.610

SERUM/PLASMA/HUMAN MILK ASSAY



(ng/mL)	O.D.	Average	Corrected
0	0.022 0.023	0.023	—
0.313	0.064 0.066	0.065	0.042
0.625	0.108 0.110	0.109	0.086
1.25	0.196 0.199	0.198	0.175
2.5	0.392 0.397	0.395	0.372
5	0.742 0.761	0.752	0.729
10	1.435 1.438	1.437	1.414
20	2.622 2.629	2.626	2.603

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.

CELL CULTURE SUPERNATE ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (ng/mL)	4.08	7.73	10.9	4.11	7.71	11.4
Standard deviation	0.189	0.229	0.281	0.295	0.511	0.726
CV (%)	4.6	3.0	2.6	7.2	6.6	6.4

SERUM/PLASMA/HUMAN MILK ASSAY

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	40	40	40
Mean (ng/mL)	3.97	7.96	11.4	4.12	7.68	11.4
Standard deviation	0.198	0.205	0.284	0.259	0.403	0.527
CV (%)	5.0	2.6	2.5	6.3	5.2	4.6

RECOVERY

The recovery of human Thrombospondin-2 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	101	91-108%
Serum* (n=4)	93	86-105%
EDTA plasma* (n=4)	93	86-105%
Heparin plasma* (n=4)	92	86-103%

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

LINEARITY

To assess the linearity of the assay, samples containing and/or spiked with high concentrations of human Thrombospondin-2 were serially diluted with the appropriate calibrator diluent to produce samples with values within the dynamic range of the assay.

		Cell culture media (n=4)	Serum* (n=4)	EDTA plasma* (n=4)	Heparin plasma* (n=4)	Human milk (n=3)
1:2	Average % of Expected	101	100	99	97	106
	Range (%)	98-104	95-107	97-100	91-101	104-109
1:4	Average % of Expected	100	103	101	98	110
	Range (%)	98-102	101-108	98-104	94-102	104-115
1:8	Average % of Expected	99	105	102	98	130
	Range (%)	93-103	100-112	99-105	93-102	———
1:16	Average % of Expected	103	107	105	103	———
	Range (%)	95-112	99-113	101-110	99-110	———

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

SENSITIVITY

Ninety-one assays were evaluated and the minimum detectable dose (MDD) of human Thrombospondin-2 ranged from 0.008-0.068 ng/mL. The mean MDD was 0.025 ng/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 Thrombospondin-2 produced at R&D Systems®.

SAMPLE VALUES

Serum/Plasma - Samples from apparently healthy volunteers were evaluated for the presence of human Thrombospondin-2 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=36)	32.9	14.3-56.1	10.4
EDTA plasma (n=36)	31.2	15.6-49.1	8.82
Heparin plasma (n=36)	31.4	13.8-51.2	8.90

Cell Culture Supernates:
Human peripheral blood cells (1 x 10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal bovine serum, 50 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed and assayed for human Thrombospondin-2. No detectable levels were observed.

IMR-90 human lung fibroblasts were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate until confluent. An aliquot of the cell culture supernate was removed, assayed for human Thrombospondin-2, and measured 0.605 ng/mL.

MDA-MB-231 human breast cancer cells were cultured in Leibovitz L-15 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine. An aliquot of the cell culture supernate was removed, assayed for human Thrombospondin-2, and measured 0.535 ng/mL.

MRC-5 human embryonic lung fibroblasts were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, and 1.0 mM sodium pyruvate. An aliquot of the cell culture supernate was removed, assayed for human Thrombospondin-2, and measured 1.69 ng/mL.

Human Milk - Three human breast milk samples were assayed for human Thrombospondin-2 and ranged from 1.50-2.17 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Thrombospondin-2.

The factors listed below were prepared at 200 ng/mL in calibrator diluent, and assayed for cross-reactivity. Preparations of the following factors at 200 ng/mL in a mid-range recombinant human Thrombospondin-2 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Thrombospondin-1
Thrombospondin-3
Thrombospondin-4 (NS0-expressed)

Recombinant human Thrombospondin-4 (CHO cell-expressed) cross-reacts approximately 0.5% in this assay.

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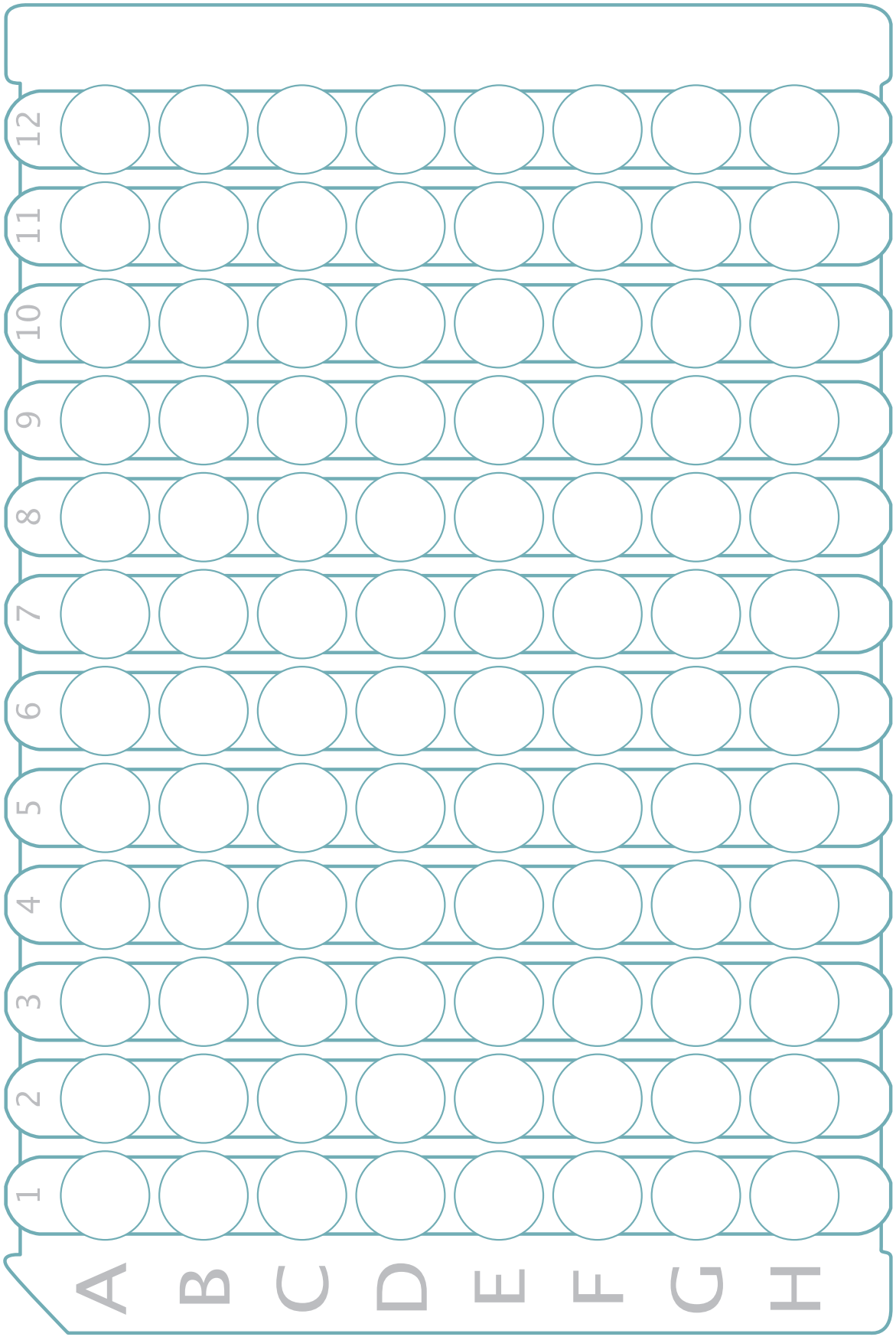
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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