Quantikine® ELISA

Human Serpin C1/Antithrombin-III Immunoassay

Catalog Number DSPC10

For the quantitative determination of human Serpin C1 concentrations in cell culture supernates, serum, plasma, urine, 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

Serpin C1, also known as Antithrombin-III, is a 58 kDa extracellular glycoprotein that belongs to the Serpin superfamily of serine protease inhibitors (1). Serpin C1 functions principally as a blood anticoagulant but also modulates inflammatory cytokine expression in the vasculature. Human Serpin C1 is synthesized in the liver and is secreted as a 432 amino acid (aa) mature protein containing four glycosylation sites (2, 3). The majority of circulating Serpin C1 (90-95%) is the fully glycosylated α-isoform while the partially glycosylated β-isoform constitutes the remaining 5-10%. The biological activity of Serpin C1 is inherently low and is enhanced up to 1000-fold in the presence of heparin or heparin-like glycosaminoglycans. The β-isoform of Serpin C1 has a higher affinity for heparin (4). Mature human Serpin C1 shares 90% aa sequence identity with mature mouse Serpin C1.

Human Serpin C1 inactivates thrombin and other blood coagulation factors, including Factors IXa, Xa, XIa, XIIa, tissue plasminogen activator, urokinase, trypsin, plasmin, and kallikrein (3, 4). Serpin C1-mediated inhibition of thrombin requires the formation of a ternary complex with heparin. Serpin C1 has also been shown to modulate inflammation (5). Direct binding of Serpin C1 to heparan sulfate expressed on endothelial cells stimulates the production of prostacyclin, an anti-inflammatory cytokine (6). In addition, Serpin C1 inhibits thrombin- and Factor Xa-mediated release of IL-6 and IL-8, and can interfere with NF-κB signaling (5-7).

In adults, Serpin C1 levels in plasma are relatively constant. Reduction of plasma Serpin C1 is observed in individuals with diabetes, sepsis, or type 1 Serpin C1 deficiency (8, 9). Neonates also exhibit low levels of Serpin C1 in plasma. Serpin C1 deficiency is associated with an increased risk of deep vein thrombosis and pulmonary embolism, which are major causes of morbidity and death (3, 10, 11). Type 1 deficiency manifests as a reduction in plasma Serpin C1 and a decrease in Serpin C1 activity. Type 2 deficiency is associated with plasma concentrations of Serpin C1 that remain close to normal while the biological activity of the protein is reduced (3, 4, 12, 13).

The Quantikine Human Serpin C1/Antithrombin-III Immunoassay is a 3.5 hour solid-phase ELISA designed to measure human Serpin C1 in cell culture supernates, serum, plasma, urine, and human milk. It contains NS0-expressed recombinant human Serpin C1 and antibodies raised against the recombinant factor. This immunoassay has been shown to accurately quantitate the recombinant factor. Results obtained using natural human Serpin C1 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 naturally occurring human Serpin C1.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Serpin C1 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Serpin C1 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human Serpin C1 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 Serpin C1 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.
- Samples, controls, and standards must be pipetted within 15 minutes.
- If samples generate values higher than the highest standard, further dilute the 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.
- 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 Serpin C1 Microplate	894888	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Serpin C1.	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 Serpin C1 Standard	894890	2 vials of recombinant human Serpin C1 in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	Discard after use. Use a new standard for each assay.
Human Serpin C1 Conjugate	894889	21 mL of a polyclonal antibody specific for human Serpin C1 conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Assay Diluent RD1W	895117	11 mL of a buffered protein base with and preservatives.	
Calibrator Diluent RD5P Concentrate	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.
- 100 mL and 500 mL graduated cylinders.
- Test tubes for dilution of standards and samples.
- Human Serpin C1 Controls (optional; R&D Systems, Catalog # QC208).

PRECAUTIONS

Serpin C1 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 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 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, 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, assay immediately or aliquot and store at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

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

Serum and plasma samples require a 50,000-fold dilution due to high endogenous values. A suggested 50,000-fold dilution can be achieved by adding 10 µL of sample to 490 µL of Calibrator Diluent RD5P (diluted 1:5)*. Then add 10 µL of the diluted sample to 490 µL Calibrator Diluent RD5P (diluted 1:5). Complete the 50,000-fold dilution by adding 10 µL of the diluted sample to 190 µL Calibrator Diluent RD5P (diluted 1:5).

Cell culture supernate and urine samples require a 2-fold dilution due to matrix effect. A suggested 2-fold dilution is 150 µL of sample + 150 µL of Calibrator Diluent RD5P (diluted 1:5).

Human milk samples require a 200-fold dilution due to high endogenous values. A suggested 200-fold dilution can be achieved by adding 10 µL of sample to 90 µL of Calibrator Diluent RD5P (diluted 1:5). Complete the 200-fold dilution by adding 10 µL of the diluted sample to 190 µL Calibrator Diluent RD5P (diluted 1:5).

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *Serpin C1 is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

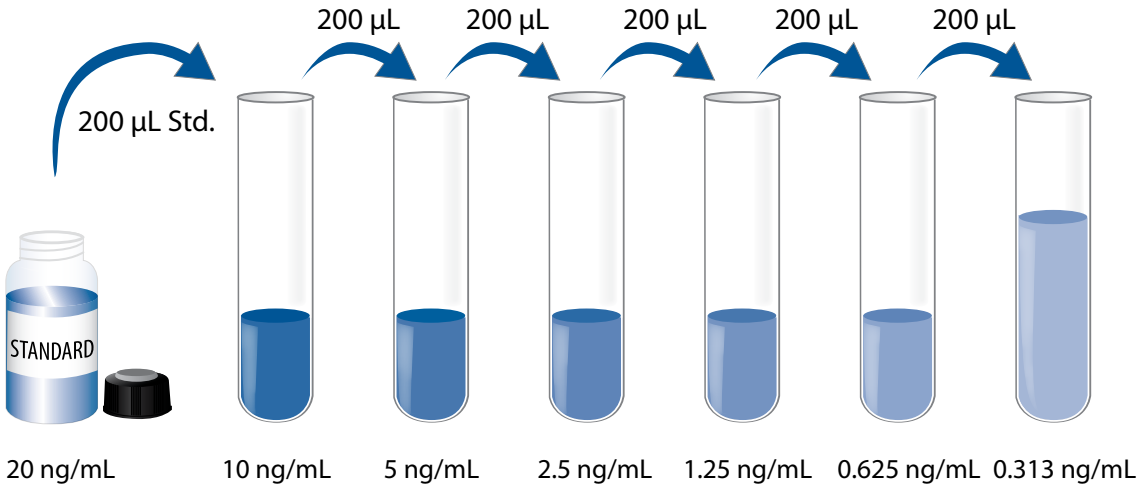
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 20 mL of Calibrator Diluent RD5P Concentrate to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5P (diluted 1:5).

Human Serpin C1 Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Serpin C1 Standard with Calibrator Diluent RD5P (diluted 1:5). This reconstitution produces a stock solution of 20 ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 5 minutes with gentle agitation prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5P (diluted 1:5) into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Human Serpin C1 Standard (20 ng/mL) serves as the high standard. Calibrator Diluent RD5P (diluted 1:5) 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.

Note: *Serpin C1 is found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

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 50 µL of Assay Diluent RD1W 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 2-8 °C.** A plate layout is provided to record standards and samples assayed.

   Note: *Samples, controls, and standards must be pipetted within 15 minutes.*
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 Serpin C1 Conjugate to each well. Cover with a new adhesive strip. Incubate for **1 hour at 2-8 °C.**
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 require dilution. See the Sample Preparation section.

CALCULATION OF RESULTS

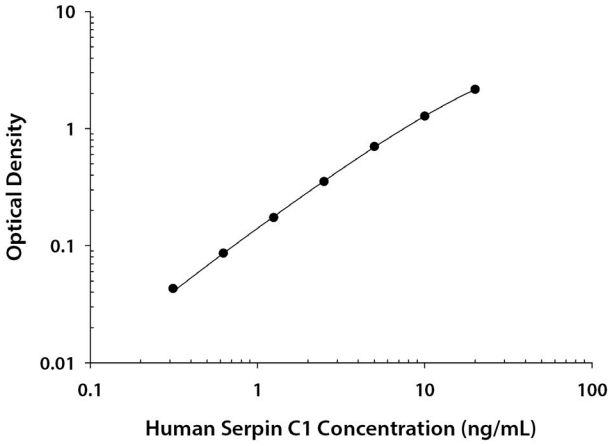
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 Serpin C1 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.



(ng/mL)	O.D.	Average	Corrected
0	0.026 0.026	0.026	—
0.313	0.067 0.070	0.069	0.043
0.625	0.110 0.113	0.112	0.086
1.25	0.194 0.205	0.200	0.174
2.5	0.375 0.382	0.379	0.353
5	0.711 0.742	0.727	0.701
10	1.258 1.342	1.300	1.274
20	2.168 2.210	2.189	2.163

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 components.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (ng/mL)	1.95	6.37	12.2	1.84	5.82	11.8
Standard deviation	0.077	0.224	0.303	0.079	0.432	0.778
CV (%)	3.9	3.5	2.5	4.3	7.4	6.6

RECOVERY

The recovery of human Serpin C1 spiked to levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	93	83-108%
Urine* (n=4)	99	92-109%

*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 Serpin C1 were diluted with 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)	Citrate plasma* (n=4)	Urine* (n=4)	Human milk* (n=4)
1:2	Average % of Expected	106	102	102	102	103	100	101
	Range (%)	101-111	99-104	101-102	100-104	99-107	95-107	99-104
1:4	Average % of Expected	104	99	104	101	104	100	101
	Range (%)	98-107	97-103	101-109	99-106	99-108	91-108	97-107
1:8	Average % of Expected	104	100	103	102	107	99	98
	Range (%)	98-108	97-106	98-111	99-109	102-115	90-110	94-104
1:16	Average % of Expected	105	101	105	103	105	100	96
	Range (%)	97-112	97-107	97-110	97-110	101-112	97-108	93-99

*Samples were diluted prior to assay.

SENSITIVITY

Thirty-three assays were evaluated and the minimum detectable dose (MDD) of human Serpin C1 ranged from 0.008-0.097 ng/mL. The mean MDD was 0.022 ng/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 highly purified NS0-expressed recombinant human Serpin C1 produced at R&D Systems.

The NIBSC/WHO Antithrombin, Plasma 3rd International Standard 08/258 was evaluated in this kit. The dose response curve of the reference reagent 08/258 parallels the Quantikine standard curve. To convert sample values obtained with the Quantikine Human Serpin C1/Antithrombin III kit to approximate NIBSC/WHO 08/258 Units, use the equation below.

NIBSC/WHO (08/258) approximate value (IU/mL) = 1 x 10^{-5} x Quantikine Serpin C1 value (ng/mL)

Note: *Based on data generated in January 2015.*

SAMPLE VALUES

Serum/Plasma/Urine/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of human Serpin C1 in this assay. No medical histories were available for the donors used in this study.

Sample Type	Mean (µg/mL)	Range (µg/mL)	Standard Deviation (µg/mL)
Serum (n=36)	107	72.0-147	18.5
EDTA plasma (n=36)	115	84.4-170	18.4
Heparin plasma (n=36)	114	71.3-172	20.6
Citrate plasma (n=36)	99.0	74.1-148	16.0

Sample Type	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Urine (n=10)	8.62	1.94-28.8	8.19
Human milk (n=10)	856	577-1692	326

Cell Culture Supernates:

HepG2 human hepatocellular carcinoma 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 until confluent. An aliquot of the cell culture supernate was removed, assayed for human Serpin C1, and measured 262 ng/mL.

Huh-7 human hepatoma cells were cultured in MEM supplemented with 10% fetal bovine serum until confluent. An aliquot of the cell culture supernate was removed, assayed for human Serpin C1, and measured 5.82 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant free and complexed human Serpin C1.

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 control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant human:

Coagulation Factor II/Thrombin
Coagulation Factor VII
Coagulation Factor X
Coagulation Factor Xa
Coagulation Factor XI
Glypican-1
Kallikrein
Matriptase
Serpin A1
Serpin A3
Serpin A4
Serpin A5
Serpin A6
Serpin A7
Serpin A9
Serpin A10
Serpin A11
Serpin A12
Serpin B2
Serpin B3
Serpin B8
Serpin B9
Serpin D1
Serpin E1
Serpin E2
Serpin F1
Serpin F2
Serpin G1
Serpin I1
Serpin I2

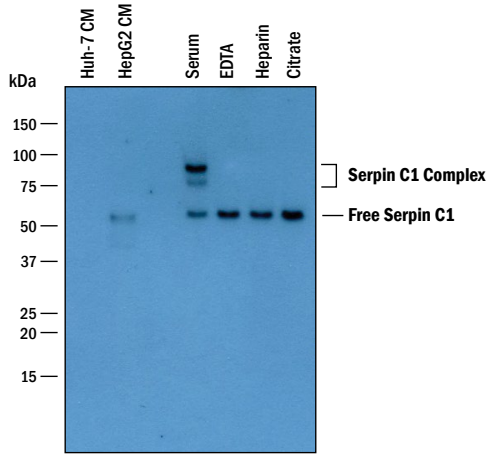
Natural proteins:

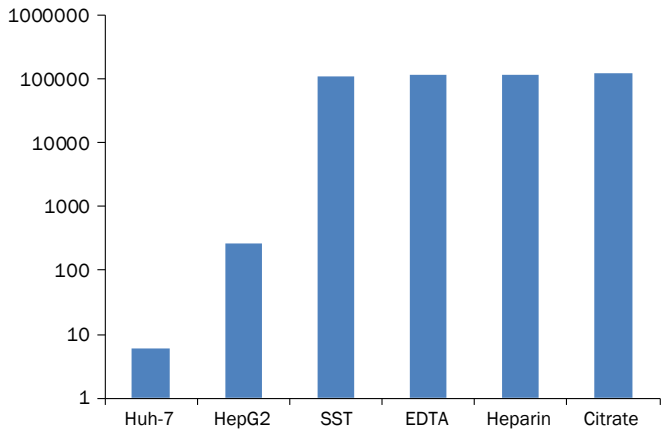
human α_2-Macroglobulin
human Plasminogen

Other factors:

Heparin
Heparin + Thrombin

Recombinant mouse Serpin C1 cross-reacts approximately 1.4% in this assay.





Human serum, EDTA, heparin, and citrate plasma samples along with Huh-7 and HepG2 conditioned media samples were analyzed by Western blot and this Quantikine ELISA kit. Samples were resolved under reducing SDS-PAGE conditions, transferred to a PVDF membrane, and immunoblotted with the detection antibody supplied in this kit. The Western blot and ELISA values for these samples correlate. In addition, the serum sample shows bands at approximately 100 kDa, which are consistent with the presence and detection of Serpin C1-Thrombin anti-Thrombin complexes. The free Serpin C1 migrates consistent with a molecular weight of approximately 58 kDa.

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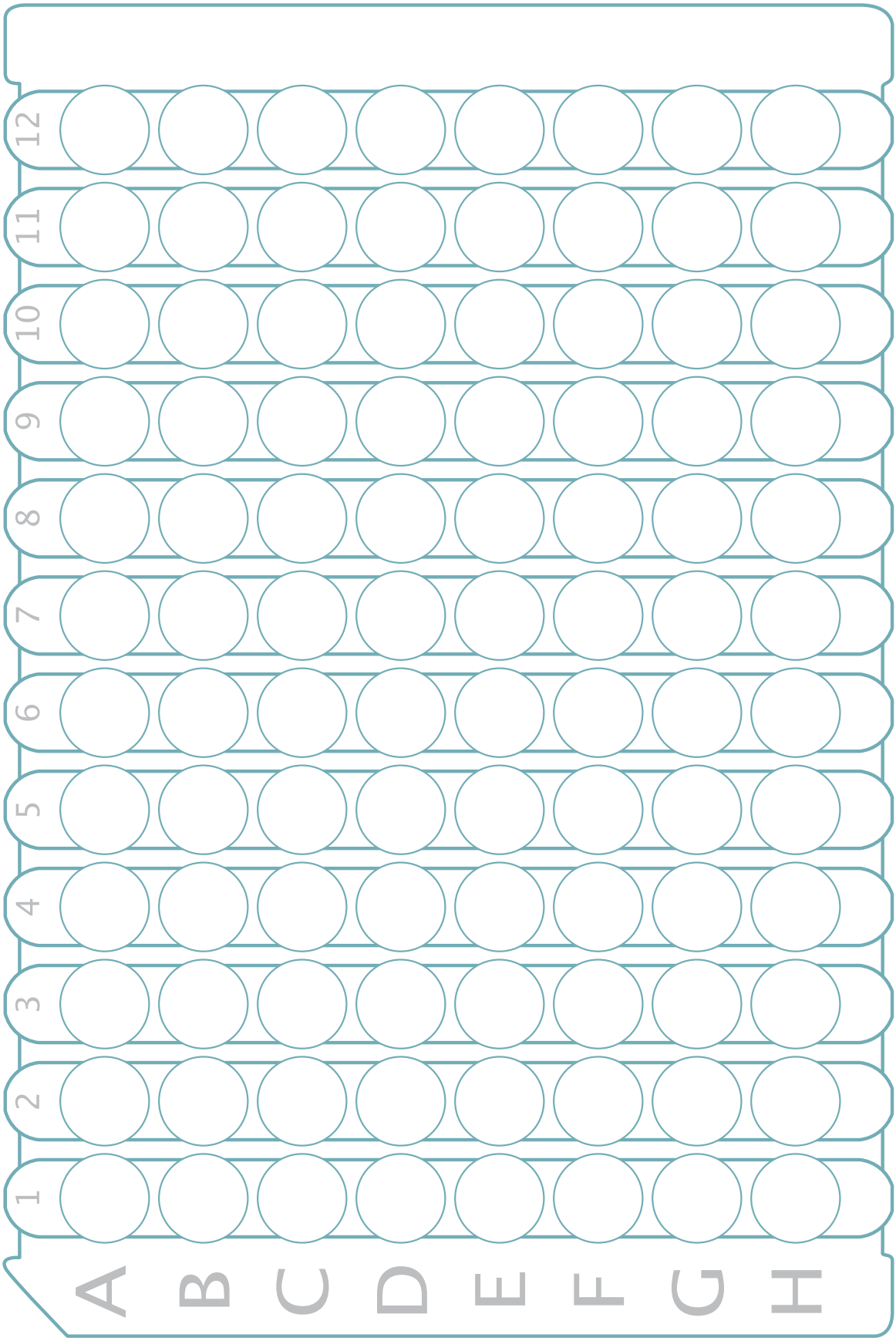
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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