Quantikine® ELISA

Human Cystatin B Immunoassay

Catalog Number DCYB00

For the quantitative determination of human Cystatin B concentrations in cell culture supernates, serum, plasma, saliva, 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

Cystatin B, also known as stefin B (gene name CSTB), is a widely expressed member of family 1 of the cystatin protease inhibitor superfamily (1, 2). This family of 11 kDa proteins lacks glycosylation and disulfide bonds (2). Cystatins A and B inhibit the activities of cysteine proteases of the papain family such as Cathepsins B, H, L and S (1-3). Human Cystatin B consists of 98 amino acids that share 79% amino acid sequence identity with mouse and rat Cystatin B (4). It is mainly found intracellularly, especially in the cytoplasm, but also at times in the nucleus and in lysosomes (1, 5, 6). In proliferating cells, it is thought to bind nuclear histones, inhibit Cathepsin L, and delay cell cycle progression by inhibiting inactivation of some transcription factors (7). In the nervous system, Cystatin B is found in neural stem cells, neurons, glia, astrocytes and neuroepithelium, and is transiently increased following seizures (5, 8). It is also found to inhibit Cathepsin K activity in rodent osteoclasts (1).

Mutations in the Cystatin B gene cause a rare syndrome, progressive myoclonus epilepsy of the Unverricht-Lundborg type, or EPM1 (1, 4, 9, 10). Most mutations cause low expression of Cystatin B, while others cause increased turnover or faulty localization within the cell (1, 6, 9). Low Cystatin B activity sensitizes cerebellar granule neurons to oxidative stress, which is thought to account for the progressive nature of EPM1 (10). Cystatin B can form multimers, which are likely to be catalytically inactive, and readily forms amyloid fibrils *in vitro* or when overexpressed (2, 11-13). Like some other amyloidogenic proteins, Cystatin B can insert into membranes and participate in pore formation *in vitro* (14). Cystatin B can also interact with amyloid-β, and Cystatin B tetramers are specifically found to inhibit amyloid-β fibril growth (12).

Serum concentrations of Cystatin B can be altered in certain cancers and autoimmune diseases. Cystatin B can inhibit cancer-promoting cathepsins and may be downregulated in plasma or serum of patients with squamous cell carcinoma of the head, neck or esophagus (15, 16). However, some cancers, such as glioblastoma, meningioma, and bladder and hepatocellular cancers, may overexpress Cystatin B (17-19). High serum Cystatin B in meningioma and colorectal cancer, and high urine Cystatin B in bladder cancer, may correlate with relapse and shorter survival (19-21). In rheumatoid arthritis, serum Cystatin B is reported to correlate with joint destruction and swelling (22).

The Quantikine Human Cystatin B Immunoassay is a 4.5 hour solid phase ELISA designed to measure Cystatin B levels in cell culture supernates, serum, plasma, saliva, urine, and human milk. It contains *E. coli*-expressed recombinant human Cystatin B and antibodies raised against the recombinant protein. Results obtained for naturally occurring human Cystatin B and recombinant human Cystatin B showed linear curves that were parallel to the standard curves obtained using the Quantikine Human Cystatin B Immunoassay standards. These results indicate that this kit can be used to determine relative mass values for natural human Cystatin B.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for Cystatin B has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Cystatin B present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for Cystatin B 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 Cystatin B 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 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
Cystatin B Microplate	894292	96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against Cystatin B.	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.*
Cystatin B Conjugate	894293	21 mL of a monoclonal antibody against Cystatin B conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Cystatin B Standard	894294	100 ng of recombinant human Cystatin B in a buffer with preservatives; lyophilized.	
Assay Diluent RD1-90	895566	11 mL of a buffered protein base with preservatives.	
Calibrator Diluent RD5-5	895485	21 mL of a buffered protein base with preservatives.	
Wash Buffer Concentrate	895003	21 mL of a 25-fold concentrated solution of buffered surfactant with preservative.	
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.
- 50 mL and 500 mL graduated cylinders.
- Test tubes for dilution of standards and samples.

PRECAUTIONS

High concentrations of Cystatin B are found in saliva. Take necessary precautions to protect kit reagents.

The Stop Solution provided with this kit is an acid solution. Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling.

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.

Saliva - Collect saliva in a tube and centrifuge for 5 minutes at 10,000 x g. Collect the aqueous layer and 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.

Human Milk - Centrifuge for 15 minutes at 1000 x g at 2-8 °C. Collect the aqueous fraction and centrifuge twice more for 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 3-fold dilution. A suggested 3-fold dilution is 50 µL of sample + 100 µL of Calibrator Diluent RD5-5 (1:4).

Saliva samples require a 200-fold dilution. A suggested 200-fold dilution can be achieved by adding 20 µL of sample to 180 µL of Calibrator Diluent RD5-5 (1:4). Complete the 200-fold dilution by adding 20 µL of the diluted sample to 380 µL of Calibrator Diluent RD5-5 (1:4).

Urine samples require a 4-fold dilution. A suggested 4-fold dilution is 50 µL of sample + 150 µL of Calibrator Diluent RD5-5 (1:4).

Human milk samples require at least a 50-fold dilution. A suggested 50-fold dilution is 10 µL of sample + 490 µL of Calibrator Diluent RD5-5 (1:4).

REAGENT PREPARATION

Bring all reagents to room temperature before use.

Note: *High concentrations of Cystatin B are found in saliva. Take necessary precautions to protect kit reagents.*

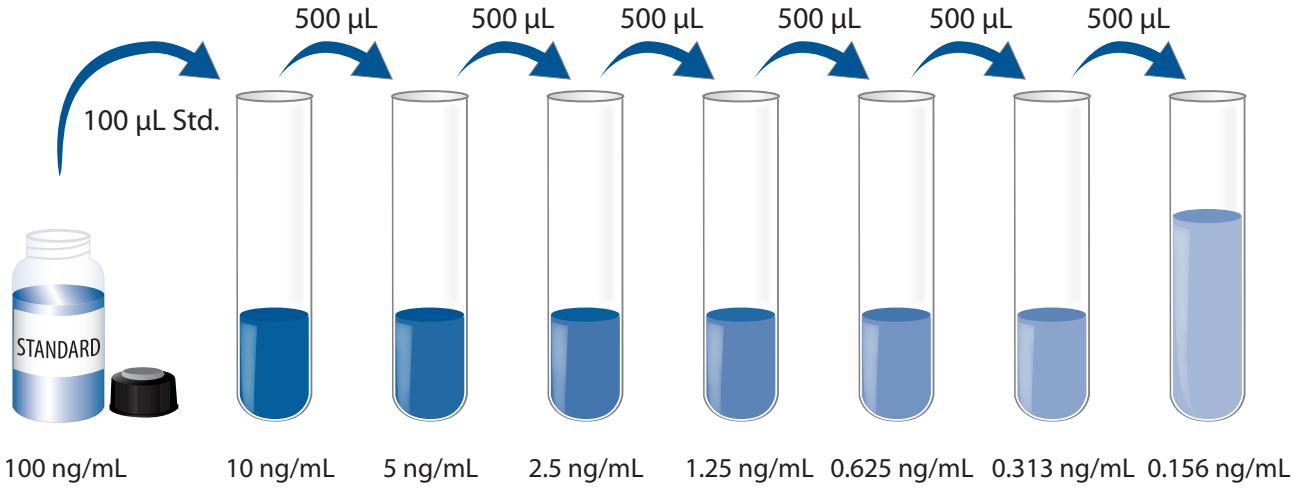
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

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 RD5-5 (1:4) - Add 10 mL of Calibrator Diluent RD5-5 to 30 mL of deionized or distilled water to yield 40 mL of diluted Calibrator Diluent RD5-5.

Cystatin B Standard - Reconstitute the Cystatin B Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 100 ng/mL. Allow the standard to sit for a minimum of 15 minutes with gentle agitation prior to making dilutions.

Pipette 900 µL of Calibrator Diluent RD5-5 (1:4) into the 10 ng/mL tube. Pipette 500 µL of Calibrator Diluent RD5-5 (1:4) into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 10 ng/mL standard serves as the high standard. Calibrator Diluent RD5-5 (1:4) 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 standards, samples, and controls be assayed in duplicate.

Note: *High concentrations of Cystatin B are found in saliva. Take necessary precautions to protect kit reagents.*

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 RD1-90 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 room temperature. 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 toweling.
6. Add 200 µL of Cystatin B 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 on the benchtop. **Protect from light.**
9. Add 50 µL of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing. 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

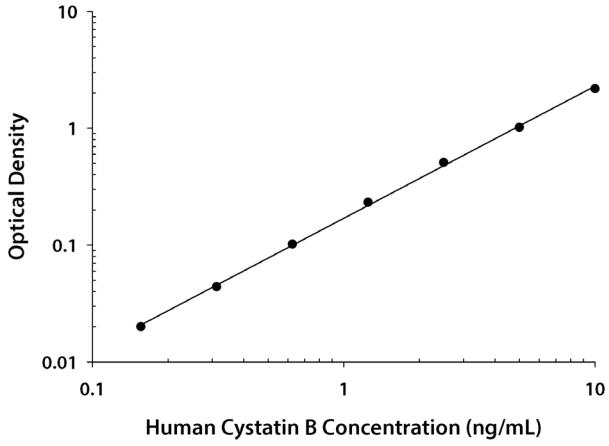
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a log/log 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 a log/log graph. The data may be linearized by plotting the log of the Cystatin B concentrations versus the log of the O.D. on a linear scale, and the best fit line can be determined by regression analysis.

If the 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	0.006 0.006	0.006	—
0.156	0.025 0.026	0.026	0.020
0.313	0.048 0.052	0.050	0.044
0.625	0.106 0.109	0.108	0.102
1.25	0.235 0.241	0.238	0.232
2.5	0.506 0.524	0.515	0.509
5	0.999 1.047	1.023	1.017
10	2.179 2.186	2.183	2.177

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.

	Intra-Assay Precision			Inter-Assay Precision		
Sample	**1**	**2**	**3**	**1**	**2**	**3**
n	20	20	20	20	20	20
Mean (ng/mL)	1.38	2.73	5.47	1.42	2.78	5.40
Standard deviation	0.049	0.082	0.164	0.148	0.191	0.263
CV (%)	3.6	3.0	3.0	10.4	6.9	4.9

RECOVERY

The recovery of Cystatin B spiked to three different levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	102	90-115%
Serum* (n=4)	93	83-103%
EDTA plasma* (n=4)	96	86-103%
Heparin plasma* (n=4)	90	81-97%

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

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of Cystatin B were serially diluted with Calibrator Diluent to produce samples with values within the dynamic range of the assay.

		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Saliva* (n=4)	Urine* (n=4)	Human milk* (n=4)
1:2	Average % of Expected	103	105	109	109	107	100	110
	Range (%)	97-108	101-110	105-114	103-113	103-112	92-110	107-113
1:4	Average % of Expected	101	108	112	112	106	98	108
	Range (%)	93-107	102-115	104-117	102-115	101-112	89-108	101-114
1:8	Average % of Expected	98	103	109	112	105	94	105
	Range (%)	88-108	100-106	94-119	106-119	97-110	86-102	100-113
1:16	Average % of Expected	89	101	101	105	99	93	99
	Range (%)	82-101	98-102	89-108	97-112	85-107	86-101	93-106

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

SENSITIVITY

Forty-four assays were evaluated and the minimum detectable dose (MDD) of Cystatin B ranged from 0.005-0.034 ng/mL. The mean MDD was 0.013 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 a highly purified *E. coli*-expressed recombinant human Cystatin B produced at R&D Systems.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine/Human Milk - Samples from apparently healthy volunteers were evaluated for the presence of Cystatin B 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)	5.41	2.47-8.46	1.57
EDTA plasma (n=36)	6.90	2.97-11.6	2.24
Heparin plasma (n=36)	5.56	2.64-9.22	1.66
Saliva (n=10)	966	57.6-2608	789
Urine (n=12)	6.41	0.764-35.4	9.60
Human milk (n=16)	153	41.5-303	87.4

Cell Culture Supernates:
Human peripheral blood leukocytes (PBLs) were cultured in DMEM supplemented with 5% bovine calf serum, 5 µM β-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. The cells were cultured unstimulated or stimulated with 10 µg/mL PHA. Aliquots of the cell culture supernates were removed on days 1 and 5 and assayed for levels of human Cystatin B.

Condition	Day 1 (ng/mL)	Day 5 (ng/mL)
Unstimulated	0.562	1.63
Stimulated	0.639	7.46

T84 human colon carcinoma cells were cultured in F-12/DMEM supplemented with 5% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. An aliquot of the cell culture supernate was removed, assayed for levels of human Cystatin B, and measured 24.4 ng/mL.

SPECIFICITY

This assay recognizes natural and recombinant human Cystatin B.

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

Recombinant human:

Cathepsin A
Cathepsin B
Cathepsin C
Cathepsin D
Cathepsin E
Cathepsin F
Cathepsin L
Cathepsin O
Cathepsin S
Cathepsin V
Cathepsin Z
Cystatin A
Cystatin C
Cystatin D
Cystatin E/M
Cystatin F
Cystatin S
Cystatin SA
Cystatin SN
Fetuin A
Fetuin B
HES-4/Cystatin A complex
HPRG
Kininogen
RTN1/Cystatin A complex

Recombinant mouse:

Cathepsin A
Cathepsin B
Cathepsin C
Cathepsin C (Active)
Cathepsin D
Cathepsin E
Cathepsin H
Cathepsin L
Cathepsin L (Pro)
Cathepsin Z
Cystatin A
Cystatin B
Cystatin C
Cystatin E/M
Fetuin A
HPRG
Kininogen

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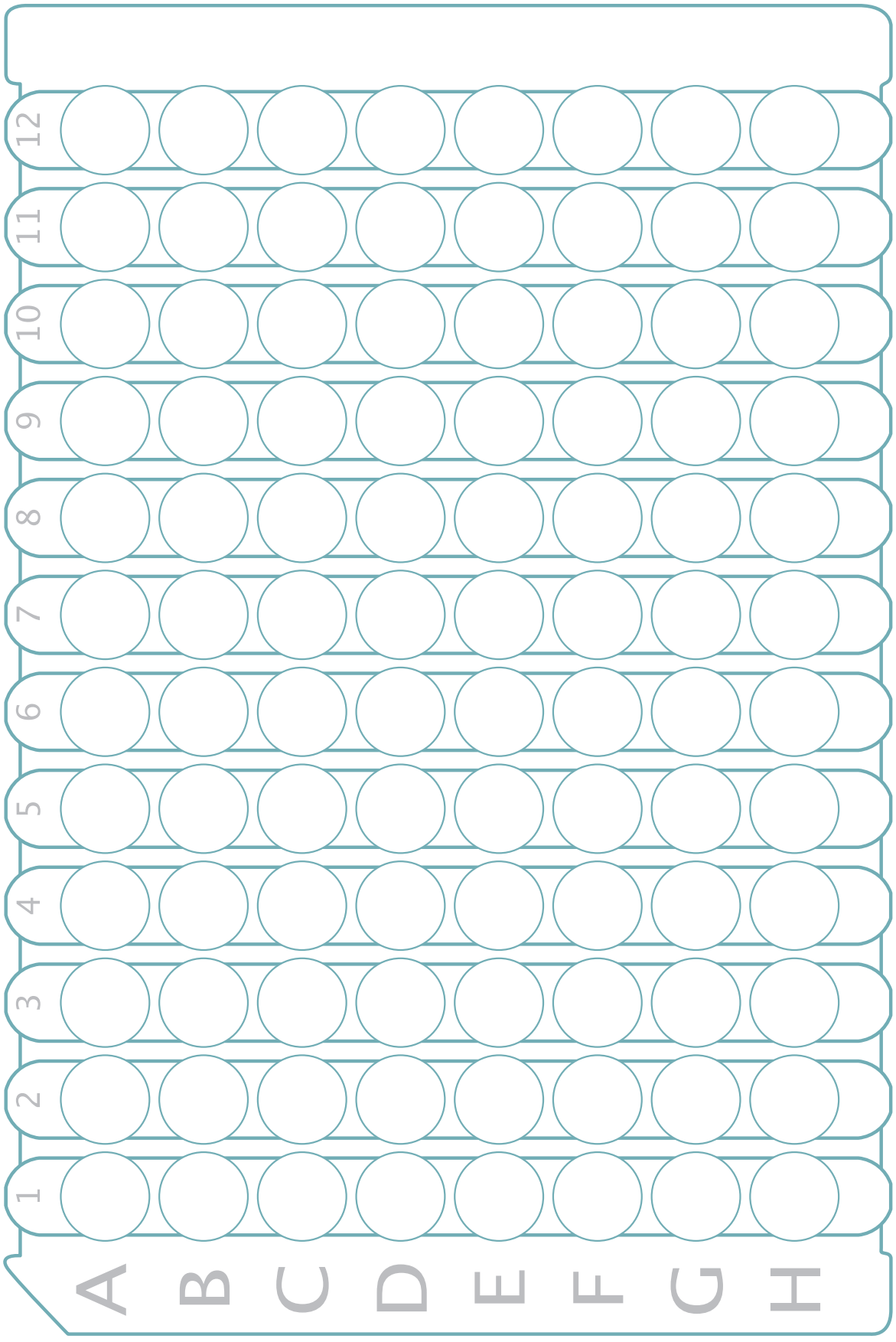
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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