Quantikine® ELISA

Human Cystatin C Immunoassay

Catalog Number DSCTC0

For the quantitative determination of human Cystatin C 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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614 McKinley Place NE, Minneapolis, MN 55413, USA
TEL: (800) 343-7475 (612) 379-2956 FAX: (612) 656-4400
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DISTRIBUTED BY:

UK & Europe | R&D Systems Europe, Ltd.
19 Barton Lane, Abingdon Science Park, Abingdon OX14 3NB, UK
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24A1 Hua Min Empire Plaza, 726 West Yan An Road, Shanghai PRC 200050
TEL: +86 (21) 52380373 FAX: +86 (21) 52371001
E-MAIL: info@RnDSystemsChina.com.cn

INTRODUCTION

Cystatin C (gene name CST3) is a secreted cysteine protease inhibitor that belongs to the cystatin superfamily (1). It is a protein of 120 amino acids and approximately 13 kDa in its non-glycosylated form; a glycosylated form is reported in rat, but not in mouse (2). Mature human Cystatin C shares 72% amino acid sequence identity with mouse and rat Cystatin C. Cystatin C is susceptible to endoprotease cleavage producing N-terminally truncated forms (1). Cysteine proteases of the papain family, such as Cathepsins B, H, K, L, and S, are the major targets for Cystatin C (3, 4).

Cystatin C is produced in all tissues and is present in all biological fluids, including plasma, urine and cerebrospinal fluid. Cystatin C is freely filtered by the glomeruli. It is then taken up by proximal tubule epithelial cells via megalin-mediated endocytosis, and is metabolized so that it does not return to the bloodstream (1, 5-7). Therefore, Cystatin C serum concentration correlates closely to the glomerular filtration rate (GFR). Its measurement in serum or plasma has been proposed as an indicator of drug nephrotoxicity that is less affected by factors such as gender, age, muscle mass and cirrhosis than creatinine (5, 7, 8). Circulating Cystatin C can, however, be increased during chronic low-level inflammation, in part due to IL-6-mediated increases in Cystatin C production (8). Conversely, the anti-inflammatory cytokines IL-10, IFN-β and IFN-γ can decrease Cystatin C expression and its circulating levels (9-11).

Cystatin C is involved in several disease processes through its regulation of cysteine protease activity (1). In humans, high circulating Cystatin C in the presence of apparently normal kidney function is associated with coronary artery and cardiovascular disease risk (1, 7, 12, 13). In a model of human aortic aneurism, deletion of mouse Cystatin C in ApoE-/- mice promotes inflammation and speeds cathepsin-mediated rupture of the arterial wall *tunica elastica* (14, 15). Circulating Cystatin C has been reported to influence tumor metastasis. Abnormally low Cystatin C levels allow cathepsin B-mediated degradation of extracellular matrix and promote tumor metastasis, while high Cystatin C levels antagonize TGF-β signaling, slowing cancer invasion and growth (16, 17). Cystatin C is an amyloidogenic protein. In humans, the L68Q variant forms dimers and oligomers more easily than the wild type protein under physiological conditions and is the cause for hereditary Cystatin C amyloid angiopathy (3, 18, 19). Cystatin C also inhibits amyloid-β deposition and protects neuronal cells from toxicity in mouse models of Alzheimer's disease (20-22).

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

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for human Cystatin C has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any Cystatin C present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for human Cystatin C 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 C 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
Human Cystatin C Microplate	893137	96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody specific for human Cystatin C.	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 Cystatin C Conjugate	893138	21 mL of a monoclonal antibody specific for human Cystatin C conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Human Cystatin C Standard	893139	Recombinant human Cystatin C in a buffer with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Assay Diluent RD1-43	895521	11 mL of a buffered protein base with preservatives. *Contains a precipitate. Mix well before and during use.*	
Calibrator Diluent RD5-24 Concentrate	895325	21 mL of a concentrate 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.
- Refrigerator (2-8 °C).
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- 100 mL and 500 mL graduated cylinders.
- Collection device for saliva samples that has no protein binding or filtering capabilities such as a Salivette® or equivalent.
- **Polypropylene** test tubes for dilution.
- Human Cystatin C Controls (optional; R&D Systems®, Catalog # QC23).

PRECAUTIONS

Cystatin C 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.

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. **Do not refreeze aliquots after use.**

Note: *Citrate plasma has not been validated for use in this assay.*

Saliva - Collect saliva using a collection device such as a Salivette or equivalent. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Note: *Saliva collector must not have any protein binding or filtering capabilities.*

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 at least a 30-fold dilution. A suggested 30-fold dilution is 20 µL of sample + 580 µL of Calibrator Diluent RD5-24 (diluted 1:5).

Saliva samples require at least a 20-fold dilution. A suggested 20-fold dilution is 30 µL of sample + 570 µL of Calibrator Diluent RD5-24 (diluted 1:5).

Human milk samples require at least a 40-fold dilution. A suggested 40-fold dilution is 15 µL of sample + 585 µL of Calibrator Diluent RD5-24 (diluted 1:5).

REAGENT PREPARATION

The conjugate must remain at 2-8 °C. Bring all remaining reagents to room temperature before use.

Note: *High concentrations of Cystatin C are found in saliva. It is recommended that a face mask and gloves be used to protect kit reagents from contamination.*

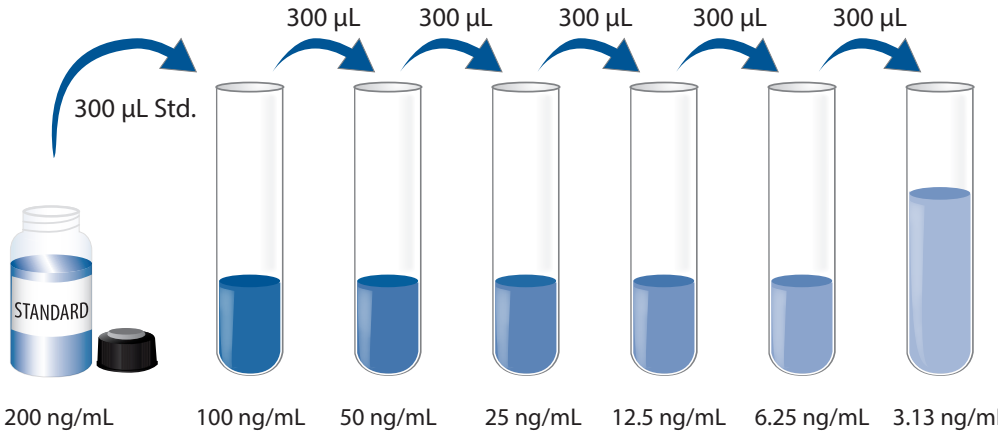
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

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 RD5-24 (diluted 1:5) - Add 20 mL of Calibrator Diluent RD5-24 Concentrate to 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5-24 (diluted 1:5).

Human Cystatin C Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Human Cystatin C 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 30 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 300 µL of Calibrator Diluent RD5-24 (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 100 ng/mL standard serves as the high standard. Calibrator Diluent RD5-24 (diluted 1:5) serves as the zero standard (0 ng/mL).



ASSAY PROCEDURE

The conjugate must remain at 2-8 °C. Bring all remaining reagents and samples to room temperature before use. It is recommended that all samples, controls, and standards be assayed in duplicate.

Note: *High concentrations of Cystatin C are 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 100 µL of Assay Diluent RD1-43 to each well. *Assay Diluent RD1-43 contains 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 3 hours at 2-8 °C.** 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 **cold** Human Cystatin C 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. **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

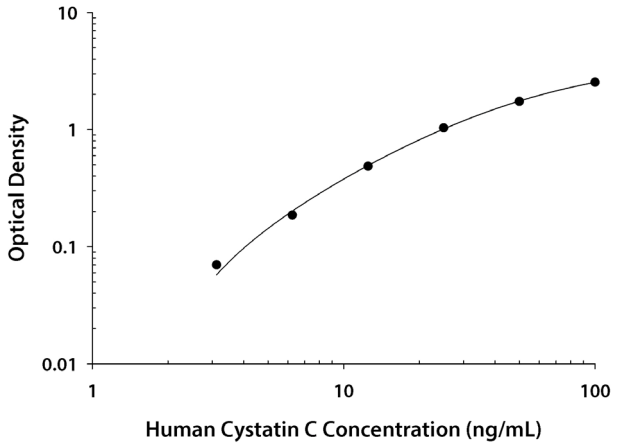
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 Cystatin C 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	0.009 0.010	0.010	—
3.13	0.076 0.082	0.079	0.069
6.25	0.193 0.198	0.196	0.186
12.5	0.491 0.501	0.496	0.486
25	1.027 1.056	1.042	1.032
50	1.699 1.796	1.748	1.738
100	2.504 2.579	2.542	2.532

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 (ng/mL)	16.2	29.9	52.6	17.2	30.8	60.9
Standard deviation	1.07	0.92	2.41	1.21	1.53	3.60
CV (%)	6.6	3.1	4.6	7.0	5.0	5.9

RECOVERY

The recovery of human Cystatin C spiked to levels throughout the range of the assay was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	96	88-102%

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of human Cystatin C were serially diluted with the 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)
1:2	Average % of Expected	102	106	105	102	100	106
	Range (%)	95-105	99-111	99-109	97-108	91-107	101-109
1:4	Average % of Expected	101	108	107	107	101	108
	Range (%)	95-108	103-114	100-114	104-109	94-109	105-110
1:8	Average % of Expected	97	105	105	104	97	103
	Range (%)	91-101	100-109	99-111	100-107	87-111	99-105
1:16	Average % of Expected	97	108	105	106	95	96
	Range (%)	88-107	107-109	99-111	98-114	89-112	87-102

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

SENSITIVITY

Fifty assays were evaluated and the minimum detectable dose (MDD) of human Cystatin C ranged from 0.030-0.227 ng/mL. The mean MDD was 0.102 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 Cystatin C produced at R&D Systems®.

This assay has been correlated to the Cystatin C reference standard supplied by the Joint Research Centre Institute for Reference Materials and Measurements (Catalog # ERM-DA471/IFCC) with a slope of 1.07 and R^2 value of 0.998.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine - Samples from apparently healthy volunteers were evaluated for the presence of human Cystatin C 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)	792	553-1257	161
EDTA plasma (n=36)	774	560-1173	155
Heparin plasma (n=36)	786	524-1284	177
Saliva (n=11)	1259	103-3184	1077
Urine (n=12)	62.9	12.6-188	43.8

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 supernate were removed and assayed for levels of human Cystatin C.

Condition	Day 1 (ng/mL)	Day 5 (ng/mL)
Unstimulated	ND	ND
Stimulated	ND	16

ND=Non-detectable

IMR-90 human lung fibroblast cells were cultured in MEM supplemented with 10% fetal bovine serum, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 0.1 mM sodium pyruvate. An aliquot of the cell culture supernate was assayed for human Cystatin C and measured 23.7 ng/mL.

MCF-7 human breast cancer cells were grown in DMEM/F12 media supplemented with 10% 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 assayed for human Cystatin C and measured 47.2 ng/mL.

HepG2 hepatocellular carcinoma cells were grown in DMEM supplemented with 10% 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 assayed for human Cystatin C and measured 264 ng/mL.

Human Milk - Two human milk samples were assayed for human Cystatin C and measured 1456 ng/mL and 2431 ng/mL, respectively.

SPECIFICITY

This assay recognizes natural and recombinant human Cystatin C.

The factors listed below were prepared at 1.0 µg/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 1.0 µg/mL in a mid-range recombinant human Cystatin C 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 L2 (V)
Cathepsin O
Cathepsin S
Cathepsin Z
Cystatin A
Cystatin B
Cystatin E/M
Cystatin F
Cystatin S
Cystatin SA
Cystatin SN
Fetuin-A
Fetuin-B
HPRG
Kininogen

Recombinant mouse:

Cathepsin A
Cathepsin B
Cathepsin C
Cathepsin C Active
Cathepsin D
Cathepsin E
Cathepsin H
Cathepsin L
Cathepsin L Proform
Cathepsin Z
Cathepsin 1
Cystatin A
Cystatin B
Cystatin E/M
Fetuin-A
HPRG
Kininogen

Some cross-reactivity was observed with the following:

Recombinant Factor	Concentration Tested	Cross-reactivity
Mouse Cystatin C	1.0 µg/mL	0.2%
Human Cystatin D	500 ng/mL	0.3%

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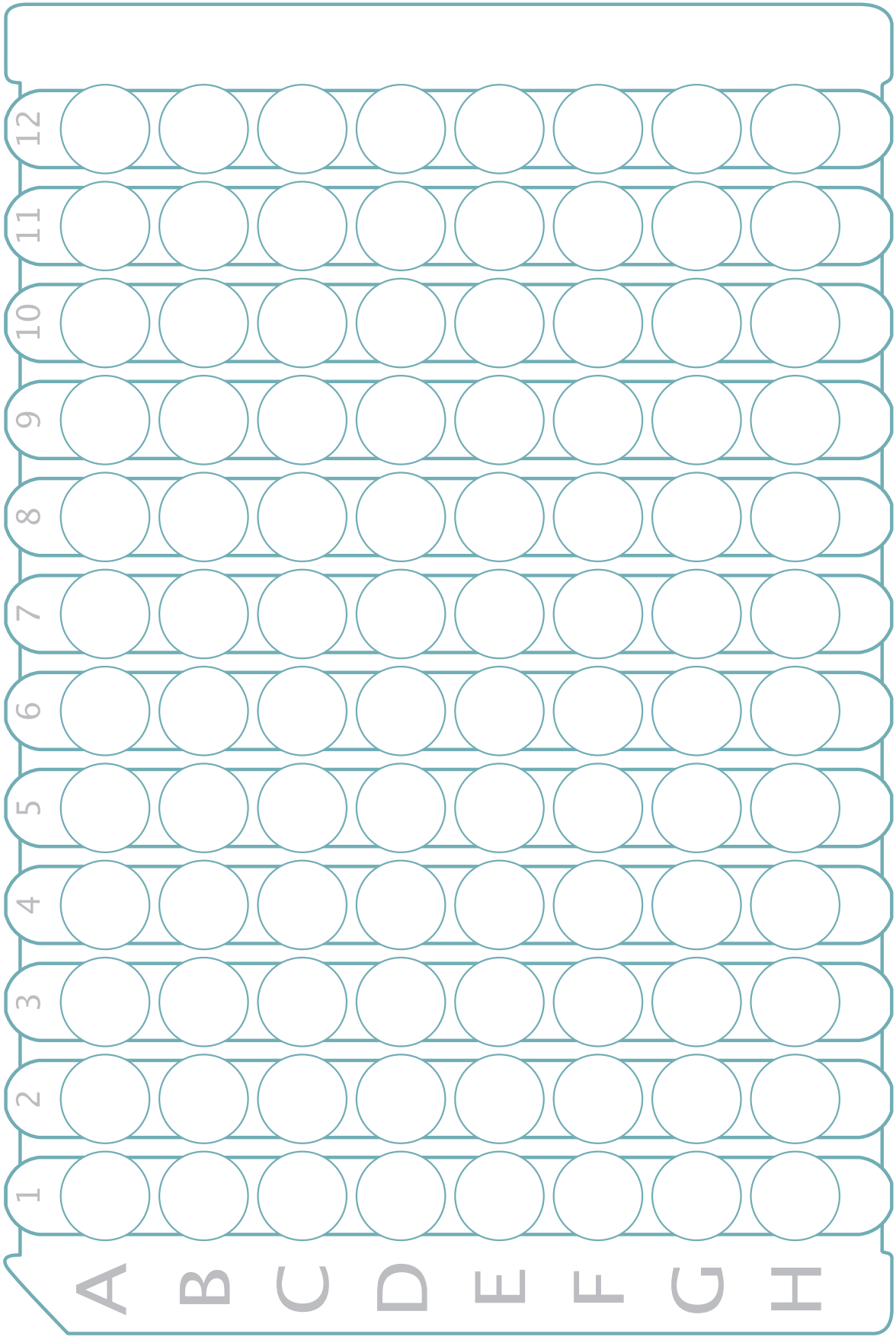
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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