Quantikine® ELISA

Mouse/Rat Cystatin C Immunoassay

Catalog Number MSCTC0

For the quantitative determination of mouse/rat Cystatin C concentrations in cell culture supernates, tissue 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

Cystatin C (gene name CST3) is a secreted, extracellular cysteine protease inhibitor that belongs to the cystatin superfamily (1-4). It is a protein of 120 amino acids (aa) and approximately 13 kDa in its non-glycosylated form; a glycosylated form is reported in rat, but not in mouse (2-4). Mouse and rat Cystatin C share 72% aa sequence identity with human Cystatin C and 88% aa sequence identity with each other. Cystatin C is susceptible to endoprotease cleavage producing N-terminally truncated forms (3, 4). Cysteine proteases of the papain family, such as Cathepsins B, H, K, L, and S, are the major targets for Cystatin C (5, 6).

Cystatin C is produced in all tissues and is present in all biological fluids. 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, 7-9). 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 (1, 7, 9). Circulating Cystatin C can, however, be increased during chronic low-level inflammation, in part due to IL-6-mediated increases in Cystatin C production (1). Conversely, the anti-inflammatory cytokines IL-10, IFN-β, and IFN-γ can decrease Cystatin C expression and its circulating levels (10-12).

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 an indicator of coronary artery and cardiovascular disease risk (1, 9, 13, 14). 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 (15, 16). 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 (1, 17, 18). Cystatin C is an amyloidogenic protein. In humans, the L68Q variant forms dimers and oligomers more easily than wild type protein under physiological conditions and is the cause for hereditary Cystatin C amyloid angiopathy (5, 19, 20). Cystatin C also inhibits amyloid-β deposition and protects neuronal cells from toxicity in mouse models of Alzheimer's disease (21-23).

The Quantikine® Mouse/Rat Cystatin C Immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse and rat Cystatin C in cell culture supernates, tissue lysates, serum, plasma, and urine. It contains NS0-expressed recombinant mouse Cystatin C and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate the recombinant factor accurately. Results obtained using natural mouse or rat Cystatin C showed dose-response 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 mouse and rat Cystatin C.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A polyclonal antibody specific for mouse/rat Cystatin C has been pre-coated onto a microplate. Standards, control, and samples are pipetted into the wells and any mouse or rat Cystatin C present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse/rat 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. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of mouse or rat Cystatin C bound in the initial step. The sample values are then read off the standard curve.

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.
- 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.
- 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.

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
Mouse/Rat Cystatin C Microplate	894073	96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody specific for mouse/rat Cystatin C.	Return unused wells to the foil pouch containing the desiccant pack. Reseal along entire edge of zip-seal. May be stored for up to 1 month at 2-8 °C.*
Mouse/Rat Cystatin C Conjugate	894074	12 mL of a polyclonal antibody specific for mouse/rat Cystatin C conjugated to horseradish peroxidase with preservatives.	May be stored for up to 1 month at 2-8 °C.*
Mouse/Rat Cystatin C Standard	894075	Recombinant mouse/rat Cystatin C in a buffered protein base with preservatives; lyophilized. *Refer to the vial label for reconstitution volume.*	
Mouse/Rat Cystatin C Control	894076	Recombinant mouse/rat Cystatin C in a buffered protein base with preservatives; lyophilized. The assay value of the control should be within the range specified on the label.	
Assay Diluent RD1W	895038	12 mL of a buffered protein solution with preservatives.	
Calibrator Diluent RD5-26 Concentrate	895525	21 mL of a concentrated buffered protein base with preservatives. *Use diluted 1:4 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	895174	23 mL of diluted hydrochloric 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.

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

- Cell Lysis Buffer 2 (R&D Systems®, Catalog # 895347).

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. Assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles.

Tissue Lysates - Cells must be lysed prior to assay as directed in the Sample Values section.

Serum - Allow blood samples to clot for 2 hours at room temperature before centrifuging for 20 minutes at 2000 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 20 minutes at 2000 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. Grossly lipemic samples are not suitable for use in this assay.*

Urine - Collect urine using a metabolic cage. Remove any particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20 °C. Avoid repeated freeze-thaw cycles. Centrifuge again before assaying to remove any additional precipitates that may appear after storage.

SAMPLE PREPARATION

Mouse serum and plasma, and rat urine samples require a 200-fold dilution. A suggested 200-fold dilution can be achieved by adding 10 µL of sample to 90 µL of Calibrator Diluent RD5-26 (diluted 1:4)*. Complete the 200-fold dilution by adding 10 µL of the diluted sample to 190 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

Rat serum and plasma samples require a 400-fold dilution. A suggested 400-fold dilution can be achieved by adding 10 µL of sample to 90 µL of Calibrator Diluent RD5-26 (diluted 1:4)*. Complete the 400-fold dilution by adding 10 µL of the diluted sample to 390 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

Mouse urine samples require a 40-fold dilution. A suggested 40-fold dilution can be achieved by adding 10 µL of sample to 390 µL of Calibrator Diluent RD5-26 (diluted 1:4)*.

*See Reagent Preparation section.

REAGENT PREPARATION

Bring all reagents to room temperature before use.

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

Mouse/Rat Cystatin C Control - Reconstitute the control with 1.0 mL of deionized or distilled water. Mix thoroughly. Assay the control undiluted.

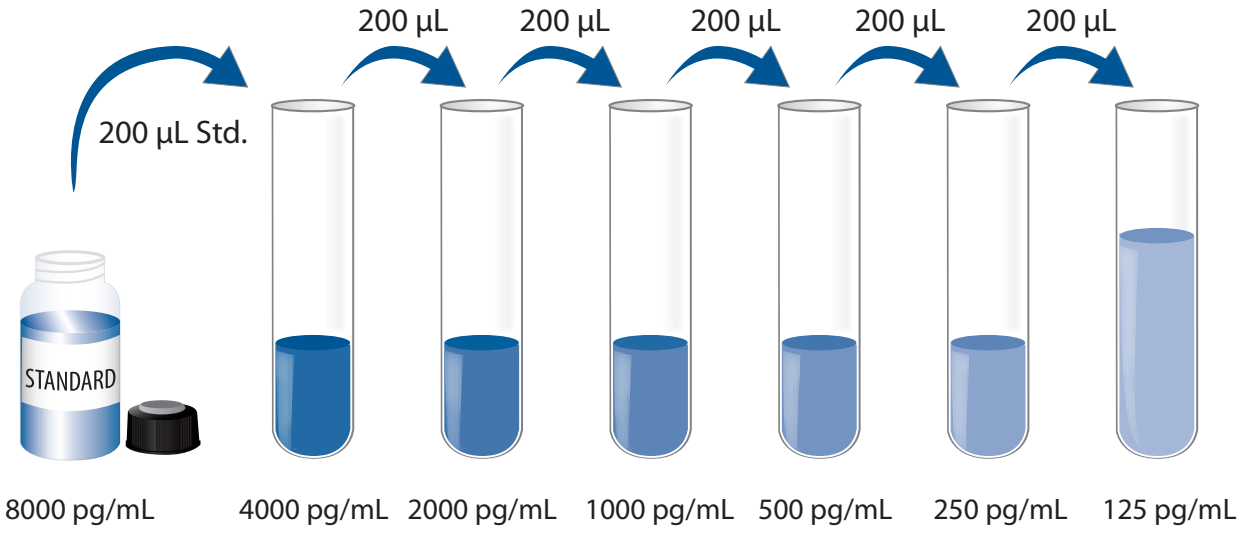
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Add 20 mL of Wash Buffer Concentrate to deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 µL of the resultant mixture is required per well.

Calibrator Diluent RD5-26 (diluted 1:4) - Add 20 mL of Calibrator Diluent RD5-26 Concentrate to 60 mL of deionized or distilled water to prepare 80 mL of Calibrator Diluent RD5-26 (diluted 1:4).

Mouse/Rat Cystatin C Standard - **Refer to the vial label for reconstitution volume.** Reconstitute the Mouse/Rat Cystatin C Standard with Calibrator Diluent RD5-26 (diluted 1:4). This reconstitution produces a stock solution of 8000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

Pipette 200 µL of Calibrator Diluent RD5-26 (diluted 1:4) into each of six tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The undiluted Mouse/Rat Cystatin C Standard (8000 pg/mL) serves as the high standard. Calibrator Diluent RD5-26 (diluted 1:4) 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 samples, control, and standards be assayed in duplicate.

Note: *Cystatin C is detectable 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, control, 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 room temperature. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process four times for a total of five 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 100 µL of Mouse/Rat Cystatin C 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 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. **Protect from light.**
9. Add 100 µL of Stop Solution to each well. 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

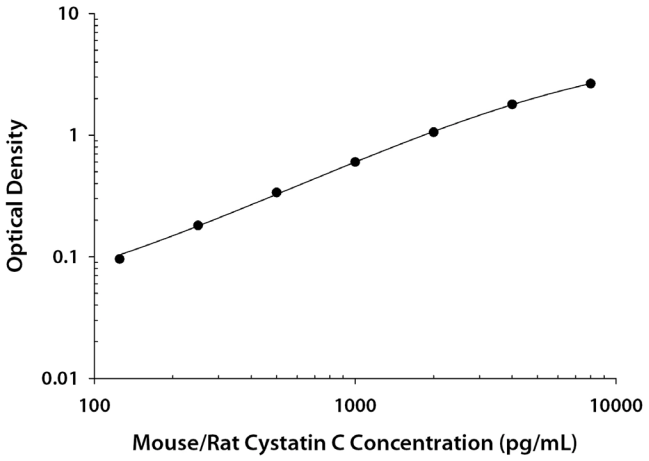
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density (O.D.).

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 mouse/rat 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.



(pg/mL)	O.D.	Average	Corrected
0	0.013 0.013	0.013	—
125	0.106 0.111	0.109	0.096
250	0.189 0.198	0.194	0.181
500	0.341 0.360	0.351	0.338
1000	0.603 0.625	0.614	0.601
2000	1.047 1.093	1.070	1.057
4000	1.802 1.804	1.803	1.790
8000	2.653 2.675	2.664	2.651

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 (pg/mL)	434	894	3065	465	942	3257
Standard deviation	14.9	29.2	85.9	43.5	51.2	213
CV (%)	3.4	3.3	2.8	9.4	5.4	6.5

RECOVERY

The recovery of mouse/rat Cystatin C spiked into cell culture media was evaluated.

Sample Type	Average % Recovery	Range
Cell culture media (n=4)	107	98-113%

SENSITIVITY

Forty-one assays were evaluated and the minimum detectable dose (MDD) of mouse/rat Cystatin C ranged from 2.47-12.9 pg/mL. The mean MDD was 3.93 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 mouse Cystatin C produced at R&D Systems®.

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of mouse or rat Cystatin C were serially diluted with calibrator diluent to produce samples with values within the dynamic range of the assay. Samples were diluted prior to assay.

Mouse Samples		Cell culture supernates (n=4)	Tissue lysates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	98	99	97	97	96	100
	Range (%)	87-101	97-100	96-98	93-100	94-101	99-101
1:4	Average % of Expected	98	97	96	95	95	102
	Range (%)	86-106	94-100	90-100	90-100	89-103	99-107
1:8	Average % of Expected	98	98	96	97	98	104
	Range (%)	88-110	93-103	87-101	92-103	94-108	103-104
1:16	Average % of Expected	99	100	95	101	92	103
	Range (%)	90-111	90-109	89-104	91-110	89-94	98-108

Rat Samples		Cell culture supernates (n=4)	Serum (n=4)	EDTA plasma (n=4)	Heparin plasma (n=4)	Urine (n=4)
1:2	Average % of Expected	92	93	97	91	94
	Range (%)	91-94	81-99	95-98	89-93	90-98
1:4	Average % of Expected	88	91	98	86	90
	Range (%)	84-92	80-100	96-99	83-89	84-95
1:8	Average % of Expected	86	92	98	85	89
	Range (%)	84-88	85-96	95-100	83-86	83-96
1:16	Average % of Expected	80	93	97	85	88
	Range (%)	80-81	87-100	91-101	81-88	83-96

SAMPLE VALUES

Serum/Plasma/Urine - Mouse and rat samples were evaluated for the presence of Cystatin C in this assay.

Mouse Samples	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=20)	447	267-638	103
EDTA plasma (n=20)	368	256-515	75.6
Heparin plasma (n=20)	333	254-495	57.6
Urine (n=20)	119	7.45-241	60.4

Rat Samples	Mean (ng/mL)	Range (ng/mL)	Standard Deviation (ng/mL)
Serum (n=20)	2111	1403-2678	360
EDTA plasma (n=20)	1542	963-1972	288
Heparin plasma (n=20)	1535	1002-2054	287
Urine (n=20)	603	239-1383	316

Cell Culture Supernates:

Organs from mice or rats were removed, rinsed in 1X PBS, and kept on ice in 1X PBS. Organs were then cut into 1-2 mm pieces and homogenized using a tissue homogenizer. Cells were seeded into media containing RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin sulfate. Cells were cultured as indicated in the tables below. Aliquots of the cell culture supernates were removed and assayed for levels of mouse/rat Cystatin C.

Mouse Tissue	(ng/mL)
Brain (1 day)	70.1
Heart (3 days)	8.4
Kidney (3 days)	29.0
Liver (3 days)	7.7
Lung (3 days)	12.6
Spleen (3 days)	14.4

Rat Tissue	(ng/mL)
Brain (18 hours)	225
Heart (18 hours)	10.2
Kidney (18 hours)	104
Lung (18 hours)	31.1
Spleen (18 hours)	8.6

3T3-L1 undifferentiated mouse embryonic fibroblast adipose-like cells (2 x 10^6 cells/T75 flask) were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and incubated for 3 days. The media was removed and 50 mL of fresh media was added and incubated for 4 additional days. An aliquot of the cell culture supernate was removed, assayed for mouse/rat Cystatin C, and measured 166 ng/mL.

3T3-L1 differentiated mouse embryonic fibroblast adipose-like cells (2 x 10^6 cells/T75 flask) were cultured in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, and incubated for 3 days. The media was removed and 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin sulfate, 1 µg/mL bovine insulin, 0.5 mM MIX, and 1 µM DEX was added and incubated for 4 additional days. An aliquot of the cell culture supernate was removed, assayed for mouse/rat Cystatin C, and measured 507 ng/mL.

SAMPLE VALUES *CONTINUED*

Tissue Lysates - Organs from mice were rinsed with 1X PBS and homogenized with a tissue homogenizer in 1X PBS. An equal volume of Cell Lysis Buffer 2 was added and tissues were lysed at room temperature for 30 minutes with gentle agitation. Debris was then removed by centrifugation. An aliquot of each tissue lysate was removed and assayed for levels of mouse Cystatin C.

Mouse Tissue	(ng/mL)
Brain	1254
Heart	210
Kidney	407
Liver	26.7
Lung	169
Spleen	127

SPECIFICITY

This assay recognizes natural and recombinant mouse and rat Cystatin C.

The factors listed below were prepared at 80 ng/mL in calibrator diluent and assayed for cross-reactivity. Preparations of the following factors at 80 ng/mL in a mid-range recombinant mouse Cystatin C control were assayed for interference. No significant cross-reactivity or interference was observed.

Recombinant mouse:
Cathepsin A
Cathepsin B
Cathepsin C
Cathepsin D
Cathepsin E
Cathepsin H
Cathepsin L
Cathepsin Z
Cystatin A
Cystatin B
Cystatin E/M

Recombinant human:
Cathepsin O
Cathepsin S
Cystatin F
Cystatin S
Cystatin SA
Cystatin SN

Recombinant human Cystatin C cross-reacts approximately 0.64% in this assay.

Recombinant human Cystatin D cross-reacts approximately 0.11% in this assay.

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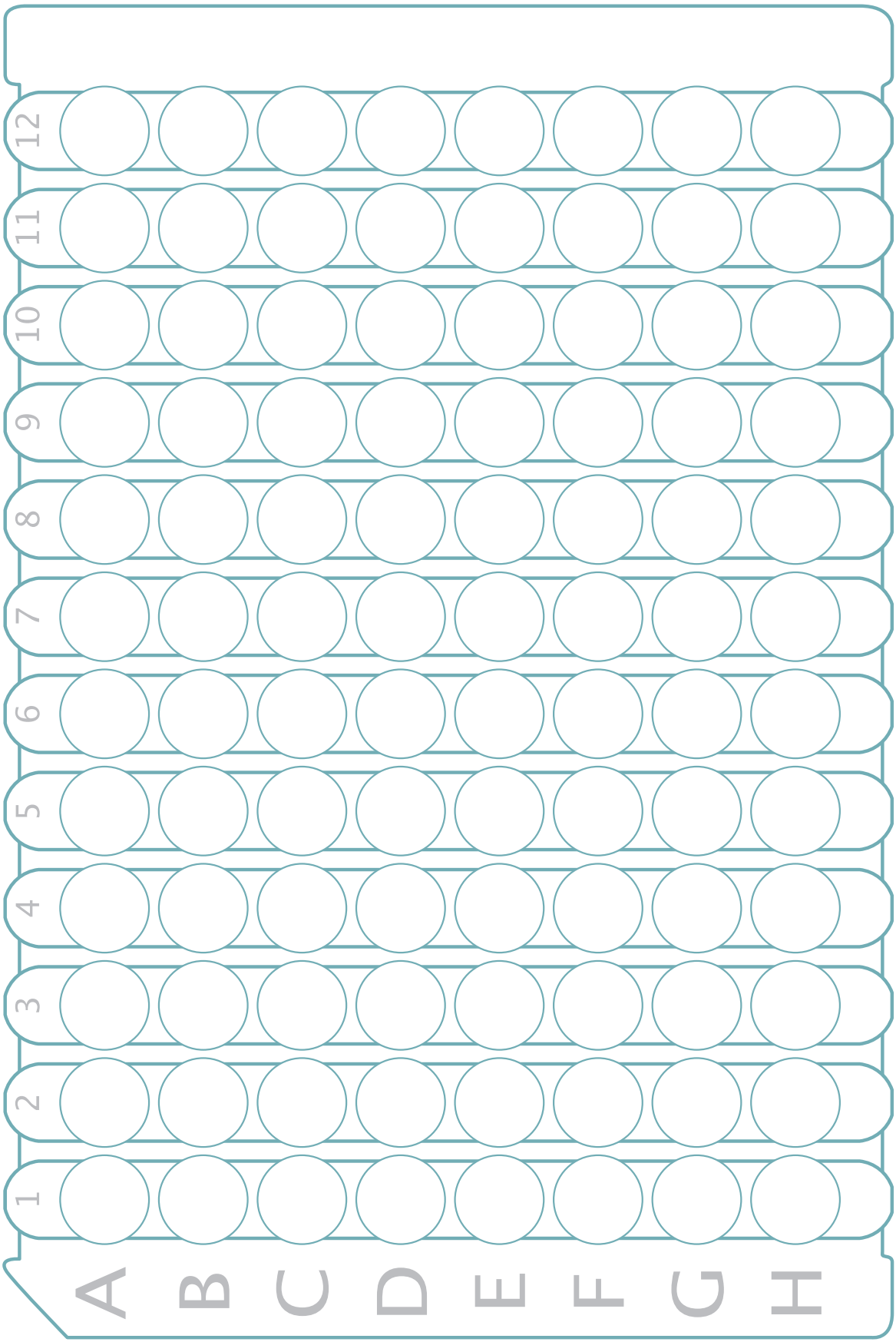
PLATE LAYOUT

Use this plate layout to record standards and samples assayed.



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