

Quantikine[®]

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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INTRODUCTION

Cystatin C is an extracellular cysteine protease inhibitor that belongs to the cystatin superfamily. Encoded by the CST3 gene on the short arm of chromosome 20, it is a non-glycosylated protein of 120 amino acids with a predicted molecular mass of 13 kDa. Major targets for Cystatin C are the cysteine proteases of the papain family. Cystatin C forms reversible 1:1 complexes with its target enzymes in competition with their substrates. As revealed by X-ray crystallography, Cystatin C has a wedge shaped protease-binding region that fits well into the substrate binding pockets of its target proteases, masking their substrate binding sites (1, 2).

Cystatin C is produced in all tissues and present in all biological fluids. Through regulating cysteine protease activity, it has been reported to be involved in many disease processes, such as inflammation and tumor metastasis (3). A single nucleotide mutation in the CST3 gene leads to hereditary Cystatin C amyloid angiopathy. This Cystatin C variant, bearing a replacement of Leucine 68 to glutamine, has a tendency to form aggregates that deposit in cerebral blood vessels (4). Cystatin C has also been found to have utility as a biomarker for renal function assessment. Because of its small size and basic pI, Cystatin C is freely filtered by the glomerulus. It is then reabsorbed by tubular epithelial cells and subsequently metabolized so that it does not return to the bloodstream. Therefore, Cystatin C serum concentration correlates closely to the glomerular clearance rate. It may be superior to creatinine due to the fact that its serum concentration is not affected by other factors, such as gender, age, and muscle mass (5, 6).

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 the Quantikine Cystatin C kit can be used to determine relative mass values for naturally occurring Cystatin C.

PRINCIPLE OF THE ASSAY

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for 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 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.
- This assay is designed to eliminate interference by proteinases and other enzymes present in biological samples. Until all enzymes have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

REAGENTS

Cystatin C Microplate (Part 893137) - 96 well polystyrene microplate (12 strips of 8 wells) coated with a mouse monoclonal antibody against Cystatin C.

Cystatin C Conjugate (Part 893138) - 21 mL of monoclonal antibody against Cystatin C conjugated to horseradish peroxidase with preservatives.

Cystatin C Standard (Part 893139) - 200 ng of recombinant human Cystatin C in a buffer with preservatives, lyophilized.

Assay Diluent RD1-43 (Part 895521) - 11 mL of a buffer with preservatives.

Calibrator Diluent RD5-24 Concentrate (Part 895325) - 21 mL of a buffered protein base with preservatives.

Wash Buffer Concentrate (Part 895003) - 21 mL of a 25-fold concentrated solution of buffered surfactant with preservatives.

Color Reagent A (Part 895000) - 12.5 mL of stabilized hydrogen peroxide.

Color Reagent B (Part 895001) - 12.5 mL of stabilized chromogen (tetramethylbenzidine).

Stop Solution (Part 895032) - 6 mL of 2 N sulfuric acid.

Plate Covers - 4 adhesive strips.

STORAGE

Unopened Kit	Store at 2 - 8° C. Do not use past kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Assay Diluent RD1-43	
	Calibrator Diluent RD5-24	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Standard	
Microplate Wells	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.*	

*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.
- 500 mL graduated cylinder.
- Collection device for saliva samples that has no protein binding or filtering capabilities such as Salivette™ or equivalent.
- **Polypropylene tubes.**
- Human Cystatin C Controls (optional; available from R&D Systems).

PRECAUTIONS

The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

Cystatin C is detectable in saliva. Take precautionary measures to prevent contamination of kit reagents while running this assay.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Serum - Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000 x g. Remove serum and assay immediately or aliquot and store samples at $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles.

Plasma - Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000 x g within 30 minutes of collection. Assay immediately or aliquot and store samples at $\leq -20^{\circ}$ 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 $\leq -20^{\circ}$ 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 $\leq -20^{\circ}$ C. Avoid repeated freeze-thaw cycles. For more information on collecting and handling urine specimens, refer to the National Committee for Clinical Laboratory Standards (NCCLS) publication GP16-T.

Human Milk - Centrifuge for 15 minutes at 10,000 x g at 2 - 8° C. Collect the aqueous fraction and repeat this process a total of 3 times. Filter through a 0.2 μ m filter and assay immediately or aliquot and store samples at $\leq -80^{\circ}$ C. Avoid repeated freeze-thaw cycles.

SAMPLE PREPARATION

Serum and plasma samples require a minimum 30-fold dilution. A suggested 30-fold dilution is 20 μ L sample + 580 μ L Calibrator Diluent RD5-24 (1X).

Saliva samples require a minimum 20-fold dilution. A suggested 20-fold dilution is 30 μ L sample + 570 μ L Calibrator Diluent RD5-24 (1X).

Human milk samples require a minimum 40-fold dilution. A suggested 40-fold dilution is 15 μ L sample + 585 μ L Calibrator Diluent RD5-24 (1X).

Cell culture supernate samples may require dilution.

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.*

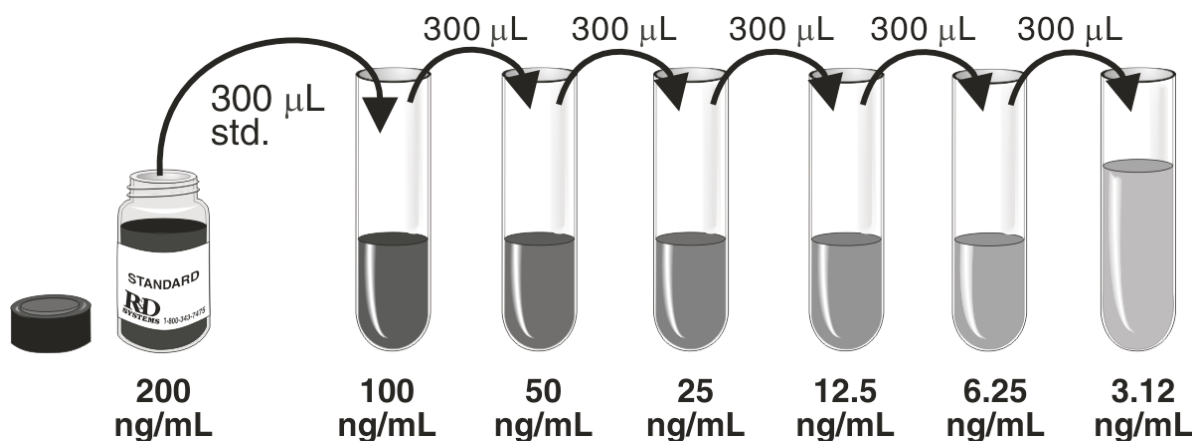
Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

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

Calibrator Diluent RD5-24 (1X) - Dilute 20 mL of Calibrator Diluent RD5-24 Concentrate into 80 mL of deionized or distilled water to prepare 100 mL of Calibrator Diluent RD5-24 (1X).

Cystatin C Standard - Reconstitute the Cystatin C Standard with 1.0 mL of 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 μ L of Calibrator Diluent RD5-24 (1X) 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. The Calibrator Diluent RD5-24 (1X) 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, reseal.
3. Add 100 µL of Assay Diluent RD1-43 to each well.
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 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.

ASSAY PROCEDURE SUMMARY

1. Prepare reagents, samples, and standards as instructed.



2. Add 100 μL Assay Diluent RD1-43 to each well.



3. Add 50 μL Standard, control, or sample* to each well.
Incubate 3 hrs. at 2 - 8° C.



4. Aspirate and wash 4 times.



5. Add 200 μL cold Conjugate to each well.
Incubate 1 hr. at 2 - 8° C.



6. Aspirate and wash 4 times.



7. Add 200 μL Substrate Solution to each well.
Incubate 30 min. at RT.
Protect from light.



8. Add 50 μL Stop Solution to each well.
Read at 450 nm within 30 min.
 λ correction 540 or 570 nm

*Samples may require dilution. See Sample Preparation section.

CALCULATION OF RESULTS

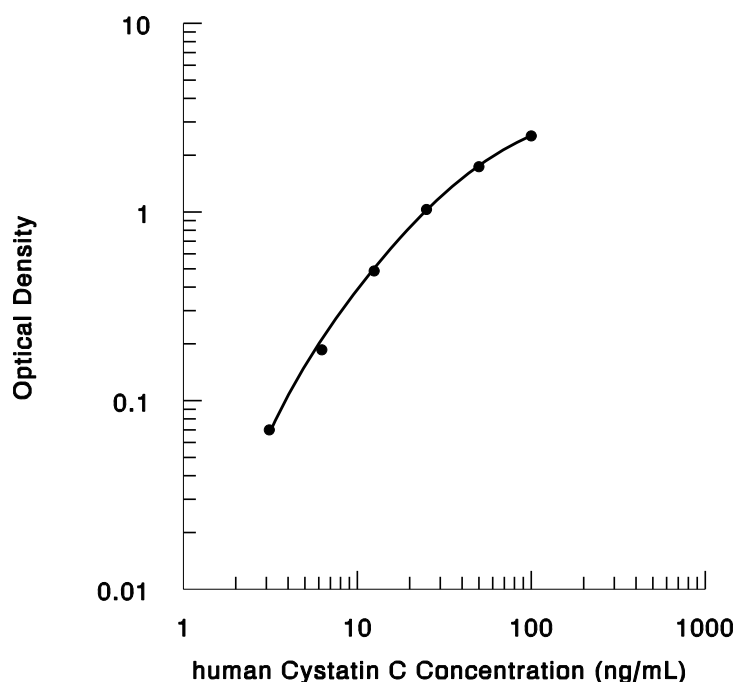
Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Create a standard curve by reducing the data using computer software capable of generating a 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 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 0.076	0.010	—
3.12	0.082 0.193	0.079	0.069
6.25	0.198 0.491	0.196	0.186
12.5	0.501 1.027	0.496	0.486
25	1.056 1.699	1.042	1.032
50	1.796 2.504	1.748	1.738
100	2.579	2.542	2.532

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.
- 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.
- 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. Wells that are green in color indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution.

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.

Sample	Intra-assay Precision			Inter-assay Precision		
	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 Cystatin C spiked to levels throughout the range of the assay was evaluated.

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

LINEARITY

To assess the linearity of the assay, samples containing high concentrations of 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)	Heparin plasma (n=4)	EDTA plasma (n=4)	Urine (n=4)	Saliva (n=4)
1:2	Average % of Expected	102	106	102	105	106	100
	Range (%)	95 - 105	99 - 111	97 - 108	99 - 109	101 - 109	91 - 107
1:4	Average % of Expected	101	108	107	107	108	101
	Range (%)	95 - 108	103 - 114	104 - 109	100 - 114	105 - 110	94 - 109
1:8	Average % of Expected	97	105	104	105	103	97
	Range (%)	91 - 101	100 - 109	100 - 107	99 - 111	99 - 105	87 - 111
1:16	Average % of Expected	97	108	106	105	96	95
	Range (%)	88 - 107	107 - 109	98 - 114	99 - 111	87 - 102	89 - 112

SENSITIVITY

Fifty assays were evaluated and the minimum detectable dose (MDD) of 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 optical density 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.

SAMPLE VALUES

Serum/Plasma/Saliva/Urine - Samples were evaluated for the presence of Cystatin C in this assay.

Sample Type	Mean (ng/mL)	Standard Deviation (ng/mL)	Range (ng/mL)
Serum (n=36)	792	161	553 - 1257
EDTA plasma (n=36)	774	155	560 - 1173
Heparin plasma (n=36)	786	177	524 - 1284
Saliva (n=11)	1259	1077	103 - 3184
Urine (n=12)	62.9	43.8	12.6 - 188

Cell Culture Supernates -

Human peripheral blood cells (1×10^6 cells/mL) were cultured in DMEM supplemented with 5% fetal calf serum, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate. Cells were cultured unstimulated or stimulated with 10 μ g/mL PHA. Aliquots of the culture supernate were removed and assayed for levels of natural Cystatin C.

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

ND= Non-detectable

IMR-90 - Cells were cultured in MEM supplemented with 10% fetal calf serum, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 0.1 mM sodium pyruvate. Aliquots of the cell culture supernate were assayed for levels of natural Cystatin C and measured 23.7 ng/mL.

MCF-7 - Cells were grown in DMEM/F12 media supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate. Aliquots of the cell culture supernate were assayed for levels of natural Cystatin C and measured 47.2 ng/mL.

HepG2 - Cells were grown in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin sulfate. Aliquots of the cell culture supernate were assayed for levels of natural Cystatin C and measured 264 ng/mL.

Breast Milk - Two human breast milk samples were assayed for levels of natural Cystatin C and measured 1456 ng/mL and 2431 ng/mL, respectively.

SPECIFICITY

This assay recognizes recombinant and natural 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 µg/mL	0.2%
Human Cystatin D	500 ng/mL	0.3%

REFERENCES

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PLATE LAYOUT

Use this plate layout as a record of standards and samples assayed.

1																	
2																	
3																	
4																	
5																	
6																	
7																	
8																	
9																	
10																	
11																	
12																	
	A	B	C	D	E	F	G	H									

NOTES

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