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User's Manual

Rat Cystatin C ELISA Kit

REF **DEIA4558**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The Rat Cystatin C ELISA is a sandwich enzyme immunoassay for the quantitative measurement of rat cystatin C.

General Description

Cystatin C or cystatin 3 (other names: gamma trace, post-gamma-globulin or neuroendocrine basic polypeptide) is a protein encoded by the CST3 gene and is synthesized in various levels by different cell-types and appears in most body fluids.

Cystatins belong to a superfamily of cysteine proteases inhibitors such as papain and Cathepsins B, H, K, L, and S. They have been found in both plants and animals.

Cystatin C, with molecular weight of 13 260 Da and composed of 120 amino acids, lacks carbohydrate and contains two disulfide bridges located near the carboxyl terminus. Cysteine proteases play an important role in protein degradation (e.g. of photoreceptor outer segments in the retinal pigment epithelium) and the balance between these proteases and their specific inhibitors is therefore of great interest.

Cystatin C level is increased in patients with malignant diseases, rheumatic diseases and related to the insufficiency of renal function. This protein appears to be a better marker than creatine. It may be especially useful in those cases where the creatinine measurement is not appropriate: for instance in liver cirrhosis, in obese, in malnourished or in patients with reduced muscle mass, too. Cystatin C measurement may be useful in the early detection of kidney disease when other parameters might still be normal. In addition to kidney dysfunction; it has been associated with an increased risk of cardiovascular disease and heart failure in older adults.

Low levels of cystatin C indicate the breakdown of the elastic laminae and, subsequently, the atherosclerosis and abdominal aortic aneurysm. The blood level of cystatin C predicts survival after one type of heart attack. On the other hand, a high level of cystatin C in the blood after a heart attack is an ominous sign because it reflects the failure of kidney to clear cystatin C from the blood into the urine. Moreover cystatin C levels are correlating with levels of triglycerides, LDL-cholesterol, BMI and the age.

Cystatin C was identified, quantitated, and localized in mouse, rat, and human retinas. In the normal adult rat retina cystatin C is present at high concentrations as it is throughout its postnatal development. Its concentration increases to a peak at the time when rat pups open their eyes and remains at a high level. It is mainly localized to the pigment epithelium, but also to some few neurons of varying types in the inner retina. Cystatin C is similarly expressed in normal mouse and human retinas.

Principles of Testing

In the Rat Cystatin C ELISA, standards, quality controls and samples are incubated in microplate wells pre-coated with polyclonal anti-rat cystatin C antibody. After 60 minutes incubation and washing, biotin labelled polyclonal anti-rat cystatin antibody is added and incubated for 60 minutes with captured cystatin C. After another washing, streptavidin-HRP conjugate is added. After 30 minutes incubation and the last washing step, the remaining conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured. The absorbance is

proportional to the concentration of cystatin C. A standard curve is constructed by plotting absorbance values against concentrations of standards, and concentrations of unknown samples are determined using this standard curve.

Reagents And Materials Provided

Antibody Coated Microtiter Strips, ready to use, 96 wells

Biotin Labelled Antibody, ready to use, 13 ml

Streptavidin-HRP Conjugate, ready to use, 13 ml

Master Standard, lyophilized, 2 vials

Quality Control HIGH, lyophilized, 2 vials

Quality Control LOW, lyophilized, 2 vials

Dilution Buffer, ready to use, 2×13 ml

Wash Solution Conc. (10×), concentrated, 100 ml

Substrate Solution, ready to use, 13 ml

Stop Solution, ready to use, 13 ml

Materials Required But Not Supplied

1. Deionized (distilled) water
2. Test tubes for diluting samples
3. Glassware (graduated cylinder and bottle) for Wash Solution (Dilution Buffer)
4. Precision pipettes to deliver 5-1000 µl with disposable tips
5. Multichannel pipette to deliver 100 µl with disposable tips
6. Absorbent material (e.g. paper towels) for blotting the microtitrate plate after washing
7. Vortex mixer
8. Orbital microplate shaker capable of approximately 300 rpm
9. Microplate washer (optional). [Manual washing is possible but not preferable.]
10. Microplate reader with 450±10 nm filter, preferably with reference wavelength 630 nm (alternatively another one from the interval 550-650 nm)
11. Software package facilitating data generation and analysis (optional)

Storage

Store the complete kit at 2-8°C. Under these conditions, the kit is stable until the expiration date (see label on the box).

Specimen Collection And Preparation

The kit measures cystatin C in rat serum.

Samples should be assayed immediately after collection or should be stored at -20°C. Mix thoroughly thawed samples just prior to the assay and avoid repeated freeze/thaw cycles, which may cause erroneous results. Avoid using hemolyzed or lipemic samples.

Dilute samples 500× with Dilution Buffer just prior to the assay in two steps as follows:

1. Dilution A (20×):

Add 5 µl of sample into 95 µl of Dilution Buffer. Mix well (not to foam). Vortex is recommended.

2. Dilution B (25×):

Add 10 µl of Dilution A into 240 µl of Dilution Buffer for duplicates to prepare final dilution (500×). **Mix well** (not to foam). Vortex is recommended.

Stability and storage: Samples should be stored at -20°, or preferably at -70°C for long-term storage. Avoid repeated freeze/thaw cycles. **Do not store the diluted samples.**

Note: It is recommended to use a precision pipette and a careful technique to perform the dilution in order to get precise results.

3. Effect of Freezing/Thawing

No decline was observed in concentration of rat cystatin C in serum samples after repeated (5×) freeze/thaw cycles. However it is recommended to avoid unnecessary repeated freezing/thawing of the samples.

Sample	Number of f/t cycles	Serum (ng/ml)
1	1x	1 016.0
	2x	1 007.4
	5x	1 064.5
2	1x	1 060.8
	2x	921.6
	5x	957.4
3	1x	664.5
	2x	740.9
	5x	754.9

4. Stability of samples stored at 2-8°C

Samples should be stored at -20°C. However, no decline in concentration of cystatin C was observed in serum samples after 14 days when stored at 2-8°C. To avoid microbial contamination, samples were treated with ε-aminocaproic acid and sodium azide, resulting in the final concentration of 0.03% and 0.1%, respectively.

Sample	Incubation Temp. Period	Serum (ng/ml)
1	-20°C	1 019.5
	2-8°C, 7 day	1 059.4
	2-8°C, 14 day	1 135.2
2	-20°C	1 159.8
	2-8°C, 7 day	1 198.1
	2-8°C, 14 day	1 188.2
3	-20°C	1 153.6
	2-8°C, 7 day	1 004.2
	2-8°C, 14 day	1 207.2

Plate Preparation

Example of a work sheet.

	strip 1+2	strip 3+4	strip 5+6	strip 7+8	strip 9+10	strip 11+12
A	Standard 25	Blank	Sample 8	Sample 16	Sample 24	Sample 32
B	Standard 12.5	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
C	Standard 6.25	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
D	Standard 3.13	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
E	Standard 1.56	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
F	Standard 0.78	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
G	QC HIGH	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
H	QC LOW	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39

Reagent Preparation

All reagents need to be brought to room temperature prior to use.

Always prepare only the appropriate quantity of reagents for your test.

Do not use components after the expiration date marked on their label.

1. Assay reagents supplied ready to use:

a. Antibody Coated Microtiter Strips

Stability and storage: Return the unused strips to the provided aluminium zip-sealed bag with desicant and seal carefully. Remaining Microtiter Strips are stable 3 months when stored at 2-8°C and protected from the moisture.

b. Biotin Labelled Antibody, Streptavidin-HRP Conjugate, Dilution Buffer, Substrate Solution, Stop Solution

Stability and storage: Opened reagents are stable 3 months when stored at 2-8°C.

2. Assay reagents supplied concentrated or lyophilized:

a. Rat Cystatin C Master Standard

Refer to Certificate of Analysis for current volume of Dilution Buffer needed for reconstitution of standard!!!

Reconstitute the lyophilized Master Standards with Dilution Buffer just prior to the assay. Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam). The resulting concentration of the rat cystatin C in the stock solution is **25 ng/ml**.

Prepare set of standards using Dilution Buffer as follows:

Volume of Standard	Dilution Buffer	Concentration
Stock	-	25 ng/ml
250 µl of stock	250 µl	12.5 ng/ml
250 µl of 12.5 ng/ml	250 µl	6.25 ng/ml
250 µl of 6.25 ng/ml	250 µl	3.13 ng/ml
250 µl of 3.13 ng/ml	250 µl	1.56 ng/ml
250 µl of 1.56 ng/ml	250 µl	0.78 ng/ml

Prepared Standards are ready to use, do not dilute them.

Stability and storage: The reconstituted standard stock solution (25 ng/ml) must be used immediately. Avoid repeated freeze/thaw cycles. **Do not store the diluted Standard solutions.**

b. Quality Controls HIGH, LOW

Refer to the Certificate of Analysis for current volume of Dilution Buffer needed for reconstitution and for current Quality Control concentration!

Reconstitute each Quality Control (HIGH and LOW) with Dilution Buffer just prior to the assay. Let it dissolve at least 15 minutes with occasional gentle shaking (not to foam).

The reconstituted Quality Controls are ready to use, do not dilute them.

Stability and storage: The reconstituted Quality Controls must be used immediately. Avoid repeated freeze/thaw cycles. Do not store the reconstituted Quality Controls.

Note: Concentration of analyte in Quality Controls need not be anyhow associated with normal and/or pathological concentrations in serum or another body fluid. Quality Controls serve just for control that the kit works in accordance with PDS and CoA and that ELISA test was carried out properly.

c. Wash Solution Conc. (10×)

Dilute Wash Solution Concentrate (10×) ten-fold in 900 ml of distilled water to prepare a 1x working solution. Example: 100 ml of Wash Solution Concentrate (10×) + 900 ml of distilled water for use of all 96-wells.

Stability and storage: The diluted Wash Solution is stable 1 month when stored at 2-8°C. Opened Wash Solution Concentrate (10×) is stable 3 months when stored at 2-8°C.

Assay Procedure

1. Pipet **100 µl** of diluted Standards, Quality Controls, Dilution Buffer (=Blank) and samples, preferably in duplicates, into the appropriate wells. See Figure for example of work sheet.

2. Incubate the plate at room temperature (ca. 25°C) for **1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.
3. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
4. Add **100 µl** of Biotin Labelled Antibody into each well.
5. Incubate the plate at room temperature (ca. 25°C) for **1 hour**, shaking at ca. 300 rpm on an orbital microplate shaker.
6. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
7. Add **100 µl** of Streptavidin-HRP Conjugate into each well.
8. Incubate the plate at room temperature (ca. 25°C) for **30 minutes**, shaking at ca. 300 rpm on an orbital microplate shaker.
9. Wash the wells 3-times with Wash Solution (0.35 ml per well). After final wash, invert and tap the plate strongly against paper towel.
10. Add **100 µl** of Substrate Solution into each well. Avoid exposing the microtiter plate to direct sunlight. Covering the plate with e.g. aluminium foil is recommended.
11. Incubate the plate for **10 minutes** at room temperature. The incubation time may be extended [up to **20 minutes**] if the reaction temperature is below than 20°C. Do not shake the plate during the incubation.
12. Stop the colour development by adding **100 µl** of Stop Solution.
13. Determine the absorbance of each well using a microplate reader set to 450 nm, preferably with the reference wavelength set to 630 nm (acceptable range: 550-650 nm). Subtract readings at 630 nm (550-650 nm) from the readings at 450 nm. The absorbance should be read within 5 minutes following step 12.

    Note: If some samples and standard/s have absorbances above the upper limit of your microplate reader, perform a second reading at 405 nm. A new standard curve, constructed using the values measured at 405 nm, is used to determine rat cystatin C concentration of off-scale standards and samples. The readings at 405 nm should not replace the readings for samples that were "in range" at 450 nm.

    Note: Manual washing: Aspirate wells and pipet 0.35 ml Wash Solution into each well. Aspirate wells and repeat twice. After final wash, invert and tap the plate strongly against paper towel. Make certain that Wash Solution has been removed entirely.

Calculation

Most microplate readers perform automatic calculations of analyte concentration. The Standard curve is constructed by plotting the mean absorbance (Y) of Standards against the known concentration (X) of Standards in logarithmic scale, using the four-parameter algorithm. Results are reported as concentration of cystatin C (ng/ml) in samples.

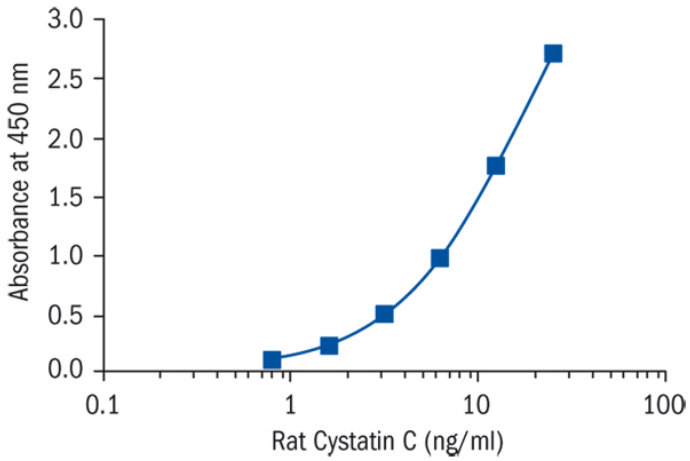
Alternatively, the logit log function can be used to linearize the standard curve, i.e. logit of the mean absorbance (Y) is plotted against log of the known concentration (X) of Standards.

The measured concentration of samples calculated from the standard curve must be multiplied by their respective dilution factor, because samples have been diluted prior to the assay, e.g. 2.7 ng/ml (from standard curve) × 500 (dilution factor) = 1350 ng/ml.

Definition of the standard

In this assay as the Standard the recombinant protein cystatin C is used. This cystatin C protein composed from 120 amino acid residues was produced in E.coli system. The apparent molecular weight is 14.93 kDa.

Typical Standard Curve



Reference Values

It is recommended that each laboratory include its own panel of control sample in the assay. Each laboratory should establish its own normal and pathological reference ranges for cystatin C levels with the assay.

Performance Characteristics

It is intended for research use only.

The total assay time is less than 3.5 hours.

The kit measures total Cystatin C in rat serum.

Assay format is 96 wells.

Quality Controls are animal serum based. No human sera are used.

Standard is recombinant protein based.

Components of the kit are provided ready to use, concentrated or lyophilized.

Detection Range

0.78-25 ng/ml

Detection Limit

0.008 ng/ml

Sensitivity

Limit of Detection (LOD) (defined as concentration of analyte giving absorbance higher than mean absorbance of blank plus three standard deviations of the absorbance of blank: $A_{blank} + 3 \times SD_{blank}$) is calculated from the real cystatin C values in wells and is 0.008 ng/ml.

Dilution Buffer is pipetted into blank wells.

Specificity

The antibodies used in this ELISA are specific for rat cystatin C. Approximately 83.4% crossreactivity with recombinant mouse cystatin C has been observed.

Sera of several mammalian species were measured in the assay. See results below.

Mammalian serum sample	Observed crossreactivity
Bovine	no
Cat	no
Dog	no
Goat	no
Hamster	no
Horse	no
Human	no
Monkey	no
Mouse	yes
Pig	no
Rabbit	no
Sheep	no

Linearity

Serum samples were serially diluted with Dilution Buffer and assayed.

Sample	Dilution	Observed (ng/ml)	Expected (ng/ml)	Recovery O/E (%)
1	-	2 041.0	-	-
	2x	1 031.0	1 020.5	101.0
	4x	593.5	510.0	116.3
	8x	283.0	255.0	111.0
2	-	2 842.0	-	-
	2x	1 420.0	1 421.0	99.9
	4x	690.0	711.0	97.2
	8x	324.0	355.0	91.3

Recovery

Serum samples were spiked with different amounts of rat cystatin C and assayed.

Sample	Observed (ng/ml)	Expected (ng/ml)	Recovery O/E (%)
1	1 027.5	-	-
	3 735.0	3 527.5	105.9
	2 342.5	2 277.5	102.9
	1 695.0	1 652.5	102.5
2	1 772.5	-	-
	4 557.5	4 272.5	106.7
	3 187.5	3 022.5	105.5
	2 512.5	2 397.5	104.8

Reproducibility

1. **Intra-assay (Within-Run) (n=8)**

Sample	Mean (ng/ml)	SD (ng/ml)	CV (%)
1	1 766.0	88.0	5.0
2	1 768.0	77.0	4.3

2. **Inter-assay (Run-to-Run) (n=6)**

Sample	Mean (ng/ml)	SD(ng/ml)	CV (%)
1	1 690.0	52.5	3.0
2	1 857.5	100.0	5.4

Precautions

1. **For professional use only**
2. Wear gloves and laboratory coats when handling immunodiagnostic materials
3. Do not drink, eat or smoke in the areas where immunodiagnostic materials are being handled
4. This kit contains components of animal origin. These materials should be handled as potentially infectious
5. Avoid contact with the acidic Stop Solution and Substrate Solution, which contains hydrogen peroxide and tetramethylbenzidine (TMB). Wear gloves and eye and clothing protection when handling these reagents. Stop and/or Substrate Solutions may cause skin/eyes irritation. In case of contact with the Stop Solution and the Substrate Solution wash skin/eyes thoroughly with water and seek medical attention, when necessary
6. The materials must not be pipetted by mouth

Trouble Shooting

1. **Weak signal in all wells**

Possible explanations:

Omission of a reagent or a step

Improper preparation or storage of a reagent

Assay performed before reagents were allowed to come to room temperature

Improper wavelength when reading absorbance

2. **High signal and background in all wells**

Possible explanations:

Improper or inadequate washing

Overdeveloping; incubation time with Substrate Solution should be decreased before addition of Stop Solution

Incubation temperature over 30°C

3. **High coefficient of variation (CV)**

Possible explanation:

Improper or inadequate washing

Improper mixing Standards, Quality Controls or samples

Limitations

1. Reagents with different lot numbers should not be mixed
2. Use thoroughly clean glassware
3. Use deionized (distilled) water, stored in clean containers
4. Avoid any contamination among samples and reagents. For this purpose, disposable tips should be used for each sample and reagent
5. Substrate Solution should remain colourless until added to the plate. Keep Substrate Solution protected from light
6. Stop Solution should remain colourless until added to the plate. The colour developed in the wells will turn from blue to yellow immediately after the addition of the Stop Solution. Wells that are green in colour indicate that the Stop Solution has not mixed thoroughly with the Substrate Solution
7. Dispose of consumable materials and unused contents in accordance with applicable national regulatory requirements