Human Cystatin C Platinum ELISA

Enzyme-linked Immunosorbent Assay for quantitative detection of human Cystatin C

Catalog Numbers BMS2279 and BMS2279TEN

Pub. No. MAN0017665 **Rev.** A.0 (30)

WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Human Cystatin C Platinum ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human Cystatin C cell culture supernatant, serum, and plasma (EDTA, citrate, heparin) have been tested with this assay..

The cystatin superfamily encompasses proteins that contain multiple cystatin-like sequences. Some of the members are active cysteine protease inhibitors, while others have lost or perhaps never acquired this inhibitory activity. There are three inhibitory families in the superfamily, including the type 1 cystatins , type 2 cystatins and the kininogens. The type 2 cystatin proteins are a class of cysteine proteinase inhibitors found in a variety of human fluids and secretions, where they appear to provide protective functions. The cystatin locus on chromosome 20 contains the majority of the type 2 cystatin genes and pseudogenes. This gene is located in the cystatin locus and encodes the most abundant extracellular inhibitor of cysteine proteases, which is found in high concentrations in biological fluids and is expressed in virtually all organs of the body. A mutation in this gene has been associated with amyloid angiopathy. Expression of this protein in vascular wall smooth muscle cells is severely reduced in both atherosclerotic and aneurysmal aortic lesions, establishing its role in vascular disease.

Principles of the test

An anti-human Cystatin C coating antibody is adsorbed onto microwells.

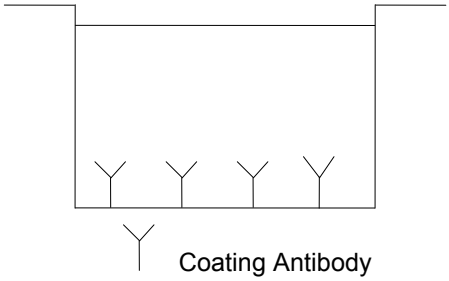

Fig. 1 Coated microwell

Cystatin C present in the sample or standard binds to antibodies adsorbed to the microwells and a HRP-conjugated anti-human Cystatin C antibody is added and binds to Cystatin C captured by the first antibody.

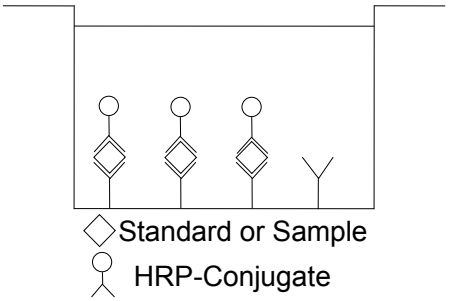

Fig. 2 First incubation

Following incubation, unbound HRP-conjugated anti-human Cystatin C antibody is removed during a wash step, and substrate solution reactive with HRP is added to the wells

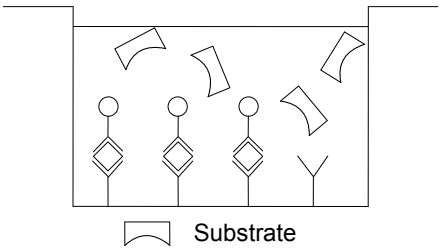

Fig. 3 Second incubation

A coloured product is formed in proportion to the amount of Cystatin C present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 7 Cystatin C standard dilutions and Cystatin C sample concentration determined

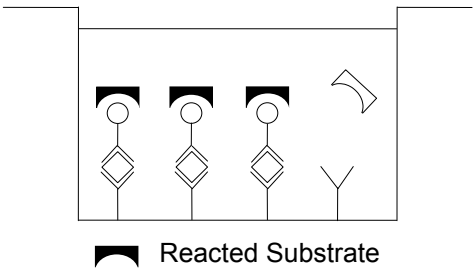

Fig. 4 Stop reaction

Reagents provided

Reagents for human Cystatin C ELISA BMS2279 (96 tests)

1 aluminum pouch with a Microwell Plate (12 strips with 8 wells each) coated with monoclonal antibody to human Cystatin C

1 vial (70 µL) HRP-Conjugate anti-human Cystatin C monoclonal antibody

2 vials human Cystatin C Standard lyophilized, 6 ng/ml upon reconstitution

1 vial (5 mL) Assay Buffer Concentrate 20x (PBS with 1% Tween™ 20, 10% BSA)

1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween™ 20)

1 vial (15 mL) Substrate Solution (tetramethyl-benzidine)

1 vial (15 mL) Stop Solution (1M Phosphoric acid)

2 Adhesive Plates

Storage instructions – ELISA kit

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.

Expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, this reagent is not contaminated by the first handling.

Sample collection and storage instructions

Cell culture supernatant, serum and plasma (citrate, heparin, EDTA) were tested with this assay. Other biological samples might be suitable for use in the assay

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic samples.

Samples should be aliquoted and must be stored frozen at –20°C to avoid loss of bioactive human Cystatin C. If samples are to be run within 24 hours, they may be stored at 2–8°C

Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

Materials required but not provided

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform regression analysis

Precautions for use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses, and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipet by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or samples.
- Rubber or disposable latex gloves should be worn while handling kit reagents or samples.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- To avoid microbial contamination or cross-contamination of reagents or samples that may invalidate the test, use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.
- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose samples and all potentially contaminated materials as if they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

Preparation of reagents

1. Buffer Concentrates should be brought to room temperature and should be diluted before starting the test procedure.
2. If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

Wash buffer (1x)

1. Pour entire contents (50 mL) of the Wash Buffer Concentrate (20x) into a clean 1000 mL graduated cylinder. Bring to final volume of 1000 mL with glass-distilled or deionized water. Mix gently to avoid foaming.
2. Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.
3. Wash Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

Assay buffer (1x)

1. Pour the entire contents (5 mL) of the Assay Buffer Concentrate (20x) into a clean 100 mL graduated cylinder. Bring to final volume of 100 mL with distilled water. Mix gently to avoid foaming.
2. Store at 2–8°C. The Assay Buffer (1x) is stable for 30 days.
3. Assay Buffer (1x) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate (20x) (mL)	Distilled Water (mL)
1–6	2.5	47.5
1–12	5.0	95.0

HRP-Conjugate

Note: The HRP-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated HRP-Conjugate solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	HRP-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

Human Cystatin C standard

1. Reconstitute human Cystatin C standard by addition of distilled water.
2. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 6 ng/mL).
3. Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.
4. After usage remaining standard cannot be stored and has to be discarded.
5. Standard dilutions can be prepared directly on the microwell plate (see "Test protocol" on page 3) or alternatively in tubes (see "External standard dilution" on page 3).

External standard dilution

1. Label 7 tubes, one for each standard point: S1, S2, S3, S4, S5, S6, S7.
2. Prepare 1:2 serial dilutions for the standard curve as follows: Pipette 225 µL of Assay Buffer into each tube.
3. Pipette 225 µL of reconstituted standard (concentration = 6 ng/mL) into the first tube, labeled S1, and mix (concentration of standard 1 = 3000 pg/mL).
4. Pipette 225 µL of this dilution into the second tube, labeled S2, and mix thoroughly before the next transfer.
5. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 5).

   Assay Buffer serves as blank.

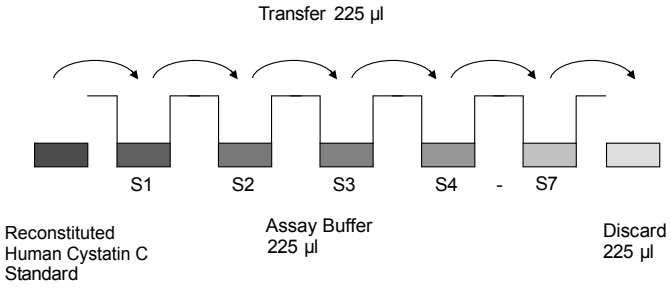

Fig. 5 Dilute standards - tubes

Test protocol

1. Predilute your serum or plasma samples before starting with the test and dilute at least 1:200 with Assay Buffer. For example:
   a. First Predilution (1:10) 10 µL + 90 µL Assay Buffer
   b. Second Predilution (1:20) 10 µL + 190 µL Assay Buffer
2. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2°-8°C sealed tightly.
3. Wash the microwell strips twice with approximately 400 µL Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration. Take care not to scratch the surface of the microwells.

   After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. Do not allow wells to dry.
4. Standard dilution on the microwell plate (Alternatively the standard dilution can be prepared in tubes - see "External standard dilution" on page 3)

   Add 100 µL of Assay Buffer (1X) in duplicate to all standard wells. Pipette 100 µL of prepared standard (see "Human Cystatin C standard" on page 3, concentration = 6 ng/mL) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 3000 pg/mL), and transfer 100 µL to wells B1 and B2, respectively (see "Test protocol" on page 3). Take care not to scratch the inner surface of the microwells. Continue this procedure 5 times, creating two rows of human Cystatin C standard dilutions ranging from 3000 to 46.9 pg/mL. Discard 100 µL of the contents from the last microwells (S7) used.

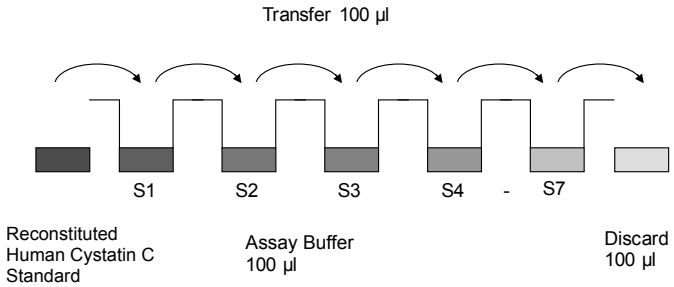

Fig. 6 Dilute standards - microwell plate

Table 1 Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (3000 pg/mL)	Standard 1 (3000 pg/mL)	Sample 1	Sample 1
B	Standard 2 (1500 pg/mL)	Standard 2 (1500 pg/mL)	Sample 2	Sample 2
C	Standard 3 (750 pg/mL)	Standard 3 (750 pg/mL)	Sample 3	Sample 3
D	Standard 4 (375 pg/mL)	Standard 4 (375 pg/mL)	Sample 4	Sample 4
E	Standard 5 (187.5 pg/mL)	Standard 5 (187.5 pg/mL)	Sample 5	Sample 5
F	Standard 6 (93.8 pg/mL)	Standard 6 (93.8 pg/mL)	Sample 6	Sample 6
G	Standard 7 (46.9 pg/mL)	Standard 7 (46.9 pg/mL)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

   In case of an external standard dilution (see "External standard dilution" on page 3), pipette 100 µL of these standard dilutions (S1 - S7) in the standard wells according to Table 1.
5. Add 100 µL of Assay Buffer (1X) in duplicate to the blank wells.
6. Add 90 µL of Assay Buffer (1X) to the sample wells.
7. Add 10 µL of each prediluted sample in duplicate to the sample wells.
8. Prepare HRP-conjugate (see "HRP-Conjugate" on page 3).
9. Add 50 µL of diluted HRP-conjugate to all well, including blank wells.
10. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, on a microplate shaker.
11. Remove adhesive film and empty wells. Wash microwell strips 3 times according to point 3 of the test protocol.

12. Pipette 100 µL of TMB Substrate Solution to all wells.
13. Incubate the microwell strips at room temperature (18° to 25°C) for about 30 min. Avoid direct exposure to intense light.

    The color development on the plate should be monitored and the substrate reaction stopped (see next point of this protocol) before positive wells are no longer properly recordable. Determination of the ideal time period for color development has to be done individually for each assay.

    It is recommended to add the stop solution when the highest standard has developed a dark blue color. Alternatively the color development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as Standard 1 has reached an OD of 0.9 – 0.95.
14. Stop the enzyme reaction by quickly pipetting 100 µL of Stop Solution into each well. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
15. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

Note: If instructions of this protocol have been followed samples have been diluted 1:2000, the concentration read from the standard curve must be multiplied by the dilution factor (x2000).

Note: In case of incubation without shaking, the obtained O.D. values may be lower than indicated below. Nevertheless, the results are still valid.

Calculation of results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 percent of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human Cystatin C concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
- To determine the concentration of circulating human Cystatin C for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human Cystatin C concentration.
- If instructions in this protocol have been followed, samples have been diluted 1:2000, the concentration read from the standard curve must be multiplied by the dilution factor (x2000).
- Calculation of samples with a concentration exceeding standard 1 will result in incorrect, low human Cystatin C levels (Hook Effect). Such samples require further external predilution according to expected human Cystatin C values with Assay Buffer (1X) in order to precisely quantitate the actual human Cystatin C level.
- It is suggested that each testing facility establishes a control sample of known human Cystatin C concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 7.

  Note: Do not use this standard curve to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

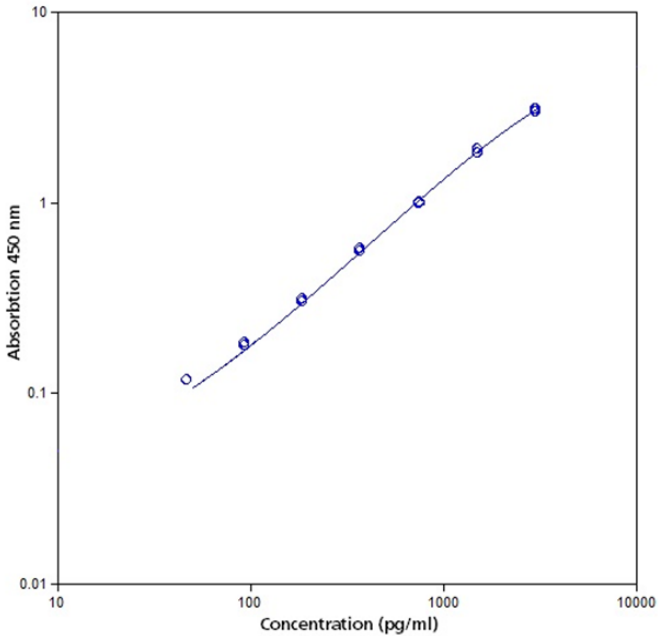

Fig. 7 Representative standard curve for human Cystatin C ELISA. Human Cystatin C was diluted in serial 2-fold steps in Assay Buffer (1X).

Table 2 Typical data using the human Cystatin C ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	Human Cystatin C Concentration (pg/mL)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	3000	3.638 3.684	3.661	0.6
2	1500	2.073 2.093	2.083	0.5
3	750	0.946 0.967	0.957	1.1
4	375	0.400 0.420	0.410	2.4
5	187.5	0.201 0.205	0.203	0.8
6	93.8	0.110 0.122	0.116	5.4
7	46.9	0.065 0.066	0.065	0.7
Blank	0	0.024 0.024	0.024	1.5

The OD values of the standard curve may vary according to the conditions of assay performance (e.g., operator, pipetting technique, washing technique, or temperature effects). Furthermore, shelf life of the kit may affect enzymatic activity and thus color intensity. Values measured are still valid.

Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.

- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.

Performance characteristics

Sensitivity

The limit of detection of human Cystatin C defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 6.9 pg/ml (mean of 3 independent assays).

Reproducibility

Intra-assay

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of serum and plasma samples containing different concentrations of human Cystatin C. 2 standard curves were run on each plate. Data below show the mean human Cystatin C concentration and the coefficient of variation for each sample. The calculated overall intra-assay coefficient of variation was 6.1%.

Table 3 The mean human Cystatin C concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human Cystatin C Concentration (ng/mL)	Coefficient of Variation (%)
1	1	1040.1	6.2
	2	1108.8	10.8
	3	1055.6	4.4
2	1	1354.4	5.0
	2	1359.7	7.4
	3	1340.7	4.5
3	1	1083.3	6.7
	2	1114.8	5.6
	3	1062.6	5.5
4	1	1167.6	6.4
	2	1187.2	5.3
	3	1138.0	5.3
5	1	1352.5	8.3
	2	1312.7	7.7
	3	1330.9	5.6
6	1	1205.8	7.3
	2	1224.0	5.2
	3	1158.9	3.3
7	1	1107.0	7.9
	2	1148.4	6.1
	3	1065.2	4.7
8	1	858.1	6.7
	2	913.1	6.7
	3	801.5	3.6

Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of serum and plasma samples containing different concentrations of human Cystatin C. 2 standard curves were run on each plate. Data below show the mean human Cystatin C concentration and the coefficient of variation calculated on 18 determinations of each sample. The calculated overall inter-assay coefficient of variation was 2.9%

Table 4 The mean human Cystatin C concentration and the coefficient of variation of each sample

Sample	Mean Human Cystatin C Concentration (ng/mL)	Coefficient of Variation (%)
1	1068.2	3.4
2	1351.6	0.7
3	1086.9	2.4
4	1164.3	2.1
5	1332.0	1.5
6	1196.2	2.8
7	1106.9	3.8
8	857.6	6.5

Dilution parallelism

Serum and plasma (EDTA, citrate, heparin) samples with different levels of human Cystatin C were analysed at serial 2 fold dilutions with 2 replicates each.

Sample matrix	Dilution	Recovery of Expected Values	
		Mean (%)	Range (%)
Serum	2000	95	86 - 100
	4000	95	86 - 102
	8000	90	80 - 101
Plasma (EDTA)	2000	98	88 - 102
	4000	93	84 - 98
	8000	83	71 - 88
Plasma (citrate)	2000	95	91 - 99
	4000	89	83 - 94
	8000	79	72 - 88
Plasma (heparin)	2000	97	94 - 100
	4000	91	83 - 106
	8000	83	62 - 92

Sample stability

Freeze-Thaw stability

Aliquots of serum were stored at -20°C and thawed 3 times, and the human Cystatin C levels determined. There was no significant loss of human Cystatin C immunoreactivity detected by freezing and thawing.

Storage stability

Aliquots of serum were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the human Cystatin C level determined after 24 h. There was no significant loss of human Cystatin C immunoreactivity detected during storage under above conditions

Specificity

The assay detects both natural and recombinant human Cystatin C. There was no cross reactivity or interference detected.

Expected values

Panels of 40 serum as well as plasma samples (EDTA, citrate, heparin) and 8 cell culture supernatant samples from randomly selected healthy donors (males and females) were tested for Cystatin C.

Sample matrix	Number of samples evaluated	Mean (ng/mL)	Range (ng/mL)	Standard deviation (ng/mL)
Serum	40	1202.9	570.4 – 2758.8	444
Plasma (EDTA)	40	1025.6	541.9 – 2347	325.2
Plasma (citrate)	40	956.1	534.3 – 2548.4	364.9
Plasma (heparin)	40	924.4	474.7– 2515.4	329.9
Cell culture supernatant	8	0.9	0.6 – 1.9	0.4

Note: The levels measured may vary with the sample collection used.

Reagent preparation summary

Wash buffer (1x)

Add Wash Buffer Concentrate 20x (50 mL) to 950 mL distilled water.

Number of Strips	Wash Buffer Concentrate (mL)	Distilled Water (mL)
1 - 6	25	475
1 - 12	50	950

Assay buffer (1x)

Add Assay Buffer Concentrate 20x (5 mL) to 95 mL distilled water.

Number of Strips	Assay Buffer Concentrate (mL)	Distilled Water (mL)
1–6	2.5	47.5
1–12	5.0	95.0

HRP-Conjugate

Make a 1:100 dilution of the concentrated HRP-Conjugate solution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

Number of Strips	HRP-Conjugate (mL)	Assay Buffer (1x) (mL)
1 - 6	0.03	2.97
1 - 12	0.06	5.94

Human Cystatin C standard

Reconstitute human Cystatin C standard with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

Test protocol summary

1. Dilute Samples 1:200.
2. Determine the number of microwell strips required.
3. Wash microwell strips twice with Wash Buffer.
4. Standard dilution on the microwell plate: Add 100 µL Assay Buffer (1X), in duplicate, to all standard wells. Pipette 100 µL prepared standard into the first wells and create standard dilutions by transferring 100 µL from well to well. Discard 100 µL from the last wells.

   Alternatively external standard dilution in tubes (see "External standard dilution" on page 3): Pipette 100 µL of these standard dilutions in the microwell strips.
5. Add 100 µL Assay Buffer (1X), in duplicate, to the blank wells.
6. Add 90 µL Assay Buffer (1X) to sample wells.
7. Add 10 µL prediluted sample in duplicate, to designated sample wells.
8. Prepare HRP-conjugate.
9. Add 50 µL of diluted HRP-conjugate to all wells
10. Cover microwell strips and incubate 2 hours at room temperature (18°-25°C) on a microplate shaker.
11. Empty and wash microwell strips 3 times with Wash Buffer.
12. Add 100 µl of TMB Substrate Solution to all wells.
13. Incubate the microwell strips for about 30 minutes at room temperature (18°C to 25°C).
14. Add 100 µl Stop Solution to all wells.
15. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed, samples have been diluted 1:2000 and the concentration read from the standard curve must be multiplied by the dilution factor (x2000).

Note: In case of incubation without shaking, the obtained O.D. values may be lower than indicated below. Nevertheless, the results are still valid.

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