biosensis® Cystatin C *Rapid*™ ELISA Kit: Human

Catalogue Number: BEK-2247-1P/2P

For the quantitative determination of Human Cystatin C (CST3) in saliva only when used as directed.

Please refer to the Sample Preparation Section for specific use instructions for each substrate application

For research use only, not for use in clinical and diagnostic procedures.

Table of Contents

1. Intended Use

The purpose of this kit is the quantitative determination of human Cystatin C in saliva. Non-validated samples may contain immunoglobulins and other components that potentially interfere with this ELISA assay. Biosensis does not assume responsibility if this kit is used for unintended purposes.

For consistent results, sample preparation is critical. Please see our sample guidelines for information on Cystatin C measurements in saliva because not all the samples tested naturally contain detectable levels of Cystatin C.

For research use only. Not for diagnostic and clinical purposes.

2. Introduction

Cystatin C (CST3, formerly gamma trace, post-gamma-globulin, or neuroendocrine basic polypeptide) is a protein encoded by the Cystatin C gene and was originally described as a constituent of normal cerebrospinal fluid (CSF) and of urine from patients with renal failure.

Cystatin C has a low molecular weight (approximately 13.3 kDa) and is removed from the bloodstream by glomerular filtration in the kidneys.

In humans, all cells with a nucleus (cell core containing the DNA) produce Cystatin C as a chain of 120 amino acids. It is found in virtually all tissues and bodily fluids. Cystatin C, which belongs to the type II cystatin gene family, is a potent inhibitor of lysosomal proteinases (enzymes from a special subunit of the cell that break down proteins) and probably one of the most important extracellular inhibitors of cysteine proteases (it prevents the breakdown of proteins outside the cell by a specific type of protein degrading enzymes). Moreover, Cystatin C is involved in network reorganization in the epileptic dentate gyrus.

The Biosensis Human Cystatin C enzyme-linked immunosorbent assay (ELISA) Kit is a sandwich ELISA that allows the preferential quantification of Cystatin C. This kit consists of a polyclonal rabbit anti-human Cystatin C capture antibody, a biotinylated goat anti-human Cystatin C detection antibody, a recombinant human Cystatin C standard, and horseradish peroxidase (HRP)-conjugated streptavidin. The addition of a substrate (3,3',5,5'-tetramethylbenzidine, TMB) yields a coloured reaction product which is directly proportional to the concentration of Cystatin C present in samples and protein standards.

3. Materials Provided and Storage Conditions

Reagent	Quantity	
	1 Plate Kit	2 Plate Kit
Human Cystatin C antibody coated 96 well microplate	12 x 8 wells	24 x 8 wells
Assay Diluent H (1x)	2 x 25 mL	4 x 25 mL
Quality Control (QC) Sample	2 Vials (see vial for amount)	4 vials (see vial for amount)
Recombinant human Cystatin C standard	2 x 4.8 ng	4 x 4.8 ng
Streptavidin-HRP (100x)	1 x 110 µL	2 x 110 µL
Cystatin C detection antibody (200x)	1 x 43 µL	2 x 43 µL
TMB substrate (1x)	1 x 11 mL	2 x 11 mL
Wash buffer (10x)	1 x 33 mL	2 x 33 mL
TMB stop solution (1x)	1 x 11 mL	2 x 11 mL
Plate sealer	Supplied	

Reagent	***Storage and Stability***
ELISA kit as supplied & unopened	12 months at 2-8°C
Reconstituted standard and QC sample	Use on same day; aliquot unused standard to prevent multiple freeze-thaw cycles and store at -20°C for 2 weeks
Diluted detection antibody and HRP conjugate (1x)	2 weeks at 2-8°C
Diluted wash buffer (1x)	2 weeks at 2-8°C

Note:

- Do not freeze the streptavidin-HRP conjugate
- Do not use kit components from other ELISA kits

4. Equipment Required but Not Supplied

- Single- and multi-channel pipettes capable of delivering 10-1000 µL volumes
- Plastic and glassware for sample collection, sample preparation and buffer preparation
- Plate shaker
- Microplate reader capable of reading absorbance at 450 nm

5. Before You Start….

- Read the entire protocol to familiarize yourself with the assay procedure
- Bring all reagents and solutions to room temperature before starting the assay
- Remove only the required amount of TMB substrate from the fridge to prevent multiple warm-ups and cool downs; keep the TMB substrate in the dark. TMB substrate should be warmed to 37°C for 30 minutes before use
- Remove the number of strips/plates required and return unused strips to the pack and reseal
- Centrifuge all kit components to collect reagents to the bottom of tubes before use
- The stop solution provided with this kit is an acid; wear protective equipment when handling
- Please visit our Technical Notes section at www.biosensis.com for further information and helpful hints on ELISA-related topics

Careful experimental planning is required to determine the total number of samples that can be assayed per plate. Refer to Appendix A for help in determining the number of microplates required to conduct an ELISA assay experiment.

6. Sample Preparation

The following sample preparations are only guidelines for specific substrates.

For unknown concentrations of Cystatin C in samples, it is important to perform several dilutions of the sample to allow the Cystatin C concentration to fall within the range of the Cystatin C standard curve (125 - 8,000 pg/mL).

Also, with unknown samples of all types it is highly recommended to run some validation experiments. This should include dilutional linearity/parallelism experiments, and spike- and recovery control tests at least at the minimum required dilution (MRD) for each sample. Parallelism and linearity of diluted samples with the standard curve demonstrates accurate quantification, as well as 80-120% recovery of spiked Cystatin C. Failure of these essential validation experiments indicates that the sample buffer, preparation, and dilution procedures will need to be optimized by the end user for consistent, accurate results with this ELISA assay. Please refer to our Technical Note #1 (ELISA Assay Validation) for further details.

Saliva

- Cystatin C has been detected in saliva at dilution ranges between 1:400 – 1:12,800. It is recommended to perform at least two dilutions when measuring the Cystatin C concentration in saliva to ensure dilution linearity

  as the saliva matrix can interfere with target detection. The optimal dilution will depend on the method of saliva collection (whole saliva, saliva supernatant, collection device).
- Please refer to McNicholas *et al* 2022 for optimized assay conditions and saliva preparation https://doi.org/10.3389/fnagi.2022.1019296.

Sample Dilutions:

The user needs to estimate the concentration of the target protein in the sample and select a proper dilution factor so that the diluted target protein concentration falls near the middle of the linear regime in the standard curve. Also, with certain growth factors and target proteins, binding proteins and location can influence the overall signal.

The following table can be used as a guideline for sample dilution with the Assay Diluent.

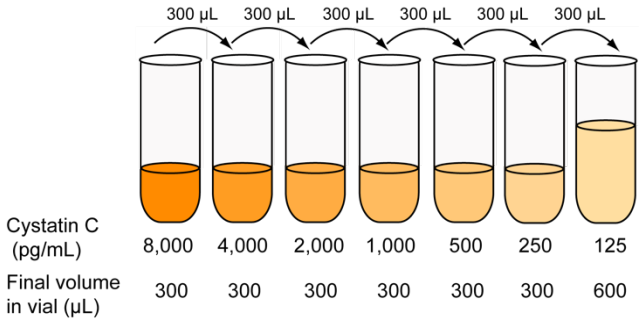
Target protein Concentration	Concentration range	Working Dilution
High	800 - 8,000 ng/mL	1:1000 - 1:8000
Medium	80 - 800 ng/mL	1:100 - 1:1000
Low	3000- 8,000 pg/mL	1:25 - 1:60

7. Preparation of Human Cystatin C standard:

- Reconstitute the lyophilized antigen standard with 600 µL of the **assay diluent H.**
- Label the vial with the reconstituted Cystatin C standard as 8,000 pg/mL. This is the first point of standard curve.
- Vortex and let stand for 15 minutes.

Perform a 1:2 serial dilution down to 125 pg/mL. The volumes used for the dilution series depends on the number of repeats per Cystatin C concentration. For duplicate measurement (100 µL per well) of each Cystatin C standard concentration, we recommend this procedure:

1. Label 6 tubes with “4000 pg/mL”, “2000 pg/mL”, “1000 pg/mL”, “500 pg/mL” “250 pg/mL” and “125 pg/mL”, respectively
2. Aliquot 300 µL of the diluent into each tube except the tube labeled “8000 pg/mL”
3. Take 300 µL from the “8000 pg/mL” tube and transfer to the “4000 pg/mL” tube.
4. Pipet up- and down and vortex to mix; to avoid foaming, use only a very brief vortex.
5. Repeat steps 3 and 4 for each consecutive concentration until the last tube “125 pg/mL” is prepared and mixed well



8. Other Reagents and Buffer Preparation

- Quality Control (QC) sample: Reconstitute the lyophilized sample with 500 µL of **assay diluent H**
- Detection antibody (250x) and streptavidin-HRP conjugate (100x): dilute two hundred and fifty-fold for detection antibody, and one hundred-fold for HRP with Assay Diluent; and prepare enough volume to add 100 µL per well.
- Wash buffer (10x): crystals may form at the bottom of the bottle; equilibrate to room temperature, mix thoroughly, and dilute ten-fold with ultrapure water; each 8-well strip requires about 25 mL of 1x wash buffer per assay.

9. Assay Procedure

We recommend that standard solution dilutions and each sample are plated in duplicate. Read entire protocol before beginning assay. TMB color developing agent must be kept warm at 37°C for 30 min before use.

1. Add 100 µL of diluted Cystatin C standards, QC sample, samples and blank (assay diluent only) to the pre-coated microplate wells
2. If available, include a negative and positive control sample in the assay procedure
3. Seal the plate (e.g., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 60 minutes
4. Discard the solution inside the wells and perform 5 washes with 1x wash buffer (200 µL per well). See the technical hints section for a detailed description of the washing procedure
5. Add 100 µL of the detection antibody (1x) into each well
6. Seal the plate (e.g., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 30 minutes
7. Discard the solution inside the wells and wash as described in step 4
8. Add 100 µL of the streptavidin-HRP conjugate (1x) into each well
9. Seal the plate (e.g., with plate sealer or parafilm) and incubate the plate on a shaker (140 rpm; 0.351 G*) for 30 minutes
10. Warm up required volume of TMB substrate in 37°C for 30 minutes before use.
    Note: TMB is light sensitive, any aliquoted volumes of TMB need to be protected from light.
11. Discard the solution inside the wells and wash as described in step 4
12. Add 100 µL of TMB into each well and incubate plate at room temperature for 10-20 minutes without shaking, in the dark
13. Stop the reaction by adding 100 µL of the stop solution into each well. Visible blue color will change to yellow. Read the absorbance at 450 nm on a plate reader.
    Note: Color will fade over time; hence, we recommend plate to be read within 5 minutes after adding the stop solution or no longer than 30 minutes after addition
14. See Appendix C for a troubleshooting guide when unexpected difficulties are encountered

* RCF= 1.12 x Orbit Radius x $(rpm/1000)^2$

CAUTION: Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.

more limited range.

10. Technical Hints

1. Do not perform dilutions within the well
2. At least duplicate measurements for each standard and sample dilution are recommended
3. Dilute samples to a Cystatin C concentration that falls within the range of the standard curve. Do not extrapolate absorbance readings
4. Avoid touching the inside surface of the wells with the pipette tip
5. Proper emptying and washing the plate is crucial for low background and non-specific binding. For manual plate washing, we recommend the following procedure:
    a. To remove liquid from the wells, place the plate on the palm of the hand and quickly invert the plate over the sink. Forcefully move the arm downwards and stop abruptly to force the liquid out of the wells. When done correctly, this technique should prevent liquid from getting onto the fingers or onto the outside of the microplate wells
    b. Blot and forcefully tap the microplate against clean paper towels for 3-5 times
    c. Wash the wells by pipetting 200 µL of wash buffer into each well and empty the wells as described in step a-b)
    d. Repeat this procedure for a total of 5 times
6. Complete removal of liquid from the wells at each step is essential for reliable results. However, avoid letting the wells dry out at any time
7. Add TMB and the stop solution to the wells in the same order
8. Ensure that all bubbles are removed and that the bottom of the plate is optically clean prior to taking the absorbance reading
9. TMB incubation times may vary based on the accuracy of diluting the ELISA kit reagents and differences in incubation time and temperature. We use a plate shaker set to 140 rpm (0.351 G) and perform all incubations at room temperature (20-25˚C, 70-75˚F) in our laboratories. Importantly, we read our plates on a microplate reader capable of measuring up to an optical density (OD) of 4. You may need to adjust the TMB incubation time if your microplate reader has a

11. Calculation of Results

1. Average the readings for each Cystatin C standard concentration, blank and sample
2. Plot a standard curve with the Cystatin C standard concentration on the x-axis and the OD at 450 nm on the y-axis
3. If values for the Cystatin C standards are adjusted for background absorbance, then subtract the blank value from the OD_{450} of the samples as well
4. Use appropriate software to reduce the data and generate a four parameter logistic (4-PL) curve-fit; do not use linear regression analysis!
5. Perform a 4-PL regression analysis to calculate the concentration of Cystatin C in the QC sample. An observed concentration within the range of 2800 – 5200 pg/mL indicates acceptable assay performance
6. Perform a 4-PL regression analysis to calculate the concentration of Cystatin C in the samples. Multiply the result by the sample dilution factor

Manual Plate Reading:
The relative $O.D._{450}$= (the $O.D._{450}$ of each well) – (the $O.D._{450}$ of Zero well).

1. The **standard curve** can be plotted as the relative $O.D._{450}$ of each standard solution (Y) vs. the respective concentration of the standard solution (X). Known concentrations of the target protein are plotted on the X-axis and the corresponding $O.D._{450}$ on the Y-axis. The standard curve should result in a graph that shows a direct relationship between target protein concentrations and the corresponding $O.D._{450}$. The greater the concentration of target protein in the sample, the higher the $O.D._{450}$.
2. **Determine concentration of target protein in unknown sample.** The target protein concentration of the samples can be interpolated from the standard curve. Draw a horizontal line to intersect with the standard curve. A vertical line dropped from this point intersects the X- axis at the concentration of antibody in the unknown sample. If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation (see step 1) to obtain the concentration in the sample, before dilution.

PC Interface Plate Reading

Enter the data into the computer program curve fitting software. Use appropriate software to reduce the data and generate a four-parameter logistic (4-PL) or 5-PL curve-fit; avoid using linear regression analysis. Perform a 4-PL regression analysis to calculate the concentration of the target analyte in the samples. Multiply the result by the sample dilution factor. See our ELISA data analysis Technical Note on our website, www.biosensis.com for more instructions on 4-PL plotting.

12. Typical Data

Standard Curve

Standard curves are provided for demonstration only (Figure 1). A standard curve has to be generated for each Cystatin C ELISA assay.

In addition, we recommend measuring common samples when performing multiple assays across several days to normalize the standard curve numbers between the various runs.

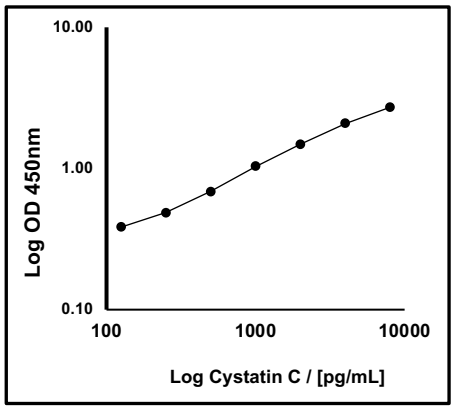


Figure 1: ExemplaryHuman Cystatin C standard curve

Typical optical densities and coefficient of Variations for diluted Cystatin C standards are summarized in the following table:

Cystatin C [pg/mL]	Mean OD Blank subtracted	CV (%)
8,000	2.00	3.9
4,000	1.33	2.1
2,000	0.81	1.7
1,000	0.46	0.6
500	0.26	3.2
250	0.12	4.6
125	0.07	4.3
Blank	0	3.2

CV: coefficient of variation

Limit of Detection

This Human Cystatin C ELISA kit detects a minimum of 80 pg/mL, defined as concentration at blank OD plus 3x standard deviation of blank OD (n=10).

Assay Precision

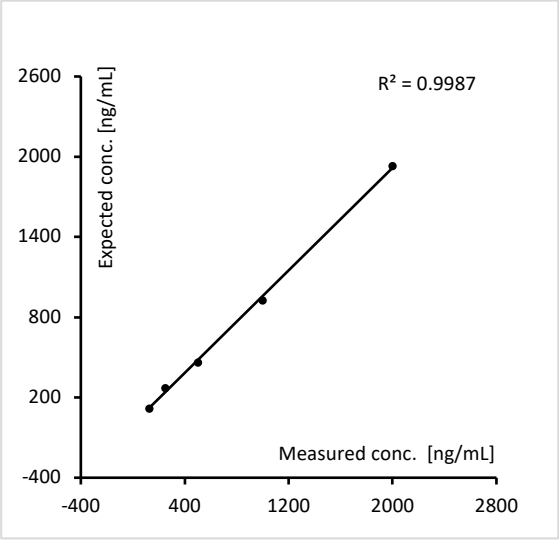
Human saliva sample was assayed in triplicate on three different days by the same operator.

Cystatin C / [µg/mL] (n=3)				Intra-Assay	Inter-Assay
	Mean concentration Dilution corrected	SD	SEM	CV	CV
Day 1	7.3	0.30	0.17	3.5%	3.4%
Day 2	7.1	0.29	0.17	7.3%	
Day 3	7.6	0.18	0.10	4.3%	

SD: standard deviation; SEM: standard error of mean; CV: coefficient of variation

Linearity of Dilution of Human Saliva

To confirm the accuracy of Cystatin C levels in normal human saliva, a spike of Cystatin C standard (4000 pg/mL) was added to a human saliva sample, that had been diluted 1:16,000 with assay diluent prior to spiking. This sample was then serially diluted by a dilution factor of 2 with the 1:16,000 diluted normal saliva sample. The measured Cystatin C values were plotted against the expected Cystatin C values.



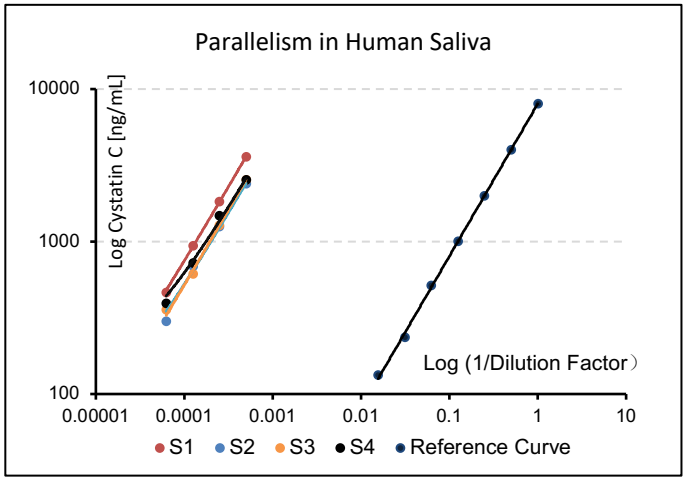
The above graph demonstrates linearity of dilution across the tested dilution range in human saliva.

Quantification of Cystatin C and Parallelism in Human Saliva samples

Human Saliva	Dilution	Cystatin C *(µg/mL)*			% of Previous Dilution
		Value	CV (%)	Mean	
S1	1:2000	5.1	6.5	5.2	-
	1:4000	5.1			100
	1:8000	4.9			98
	1:16000	5.7			116
S2	1:2000	7.2	1.7	7.3	-
	1:4000	7.3			101
	1:8000	7.5			103
	1:16000	7.4			99
S3	1:2000	5.9	8.4	5.8	-
	1:4000	5.8			98
	1:8000	6.3			109
	1:16000	5.1			81
S4	1:2000	4.8	2.2	5.0	-
	1:4000	5.0			104
	1:8000	5.5			110
	1:16000	4.8			87

Four human saliva samples were each diluted 1/2000 to 1/16,000 with the assay diluent provided in this kit and assayed in triplicates. A 4-PL regression analysis was carried out to determine mean Cystatin C levels in saliva.

The assayed four saliva samples in four dilutions ranging from 1/2000 to 1/16000 were assessed Parallelism with Cystatin C reference standard.



Spike and Recovery of Cystatin C in Human Saliva

Human saliva sample was initially diluted at 1/4000 in Assay Diluent, and then spiked with 2000 pg/mL of the supplied Cystatin C protein standard. The mixture was serially diluted in assay diluent by a factor of 2 to a final dilution of 1/32,000. for the saliva sample, and to 1000 pg/mL, 500 pg/mL, and 250 pg/mL for the Cystatin C protein.

Dilution	% previous dilution	% Recovery
1/4000	-	98%
1/8000	102%	100%
1/16000	104%	104%
1/32000	102%	107%

Specificity

There is no detectable cross-reactivity with other proteins

13. Specific References

McNicholas K, François M, Liu J-W, Doecke JD, Hecker J, Faunt J, Maddison J, Johns S, Pukala TL, Rush RA and Leifert WR (2022) Salivary inflammatory biomarkers are predictive of mild cognitive impairment and Alzheimer's disease in a feasibility study. *Front. Aging Neurosci.* 14:1019296. doi: 10.3389/fnagi.2022.1019296 https://doi.org/10.3389/fnagi.2022.1019296

14. Other Information

Please visit our website (www.biosensis.com) for a full range of Cystatin C-related research assays

Appendix A: Determining the Number of Microplate Wells Needed for ELISA Experiments

Standard curve, blank and controls:

- Standard (8,000 pg/mL, 4,000 pg/mL, 2,000 pg/mL, 1,000 pg/mL, 500 pg/mL, 250 pg/mL and 125 pg/mL): 7 wells
- Blank (0 pg/mL): 1 well

Gives a total of 8 wells used per Standard

We recommend that the standard curve and blanks be run on each separate plate for the most accurate calculations. Note that if two (or more) independent assays are run on one plate, each assay requires its own Standard curve. We also recommend that ALL samples (i.e., standards, blanks, and test subjects) be performed in duplicate at least.

Thus, for standards and blanks, 8 x 2 = **16 standard wells are required per assay.** There are 96 wells per plate – 16 wells = **80 sample wells per plate** left for test/patient samples for EACH plate with a standard curve and blank

Example: 60 patient samples, duplicate measurement at one single dilution

How many wells and plates do I need for sixty patients at one dilution with a single sample taken before and after treatment?

1. Calculate the number of test samples: 60 patients, x 2 draws each (e.g., before and after) = 120 stock samples
2. Factor in the number of repeat tests desired, in order to calculate the number of wells required. Biosensis recommends that ALL samples be run in duplicate at least.
3. Multiply the number of stock samples by the number of dilutions per sample. In this example, we will be testing EACH stock sample at a single dilution (i.e., 1:100) in duplicate, so 120 stock samples x 2 wells = 240 microplate wells required for sixty patients. In summary: 60 patients, 4 tests per patient, equals 240 total number of wells required.
4. Decide on the number of standards and blanks run on each plate. We recommend standards and blanks in duplicate for each 96-well plate. Alternatively, one standard, blank and control duplicate set can be run on every 2 plates

Running the Test:

Running the 60 patient experiment with a standard curve, blanks and controls on *each* test plate, in duplicate (our recommended option):

Single Dilution per Test Sample:

(240 wells required) / (80 wells per plate available) = 3 plates required, thus 3 full plates will be required from this Cystatin C kit to ensure enough wells for the entire sixty patient samples, tested in duplicate (two draws per patient, 1 dilution, 4 wells per patient, total of 240 wells).

Appendix B: Troubleshooting Guide

This Cystatin C ELISA kit has been developed to deliver reproducible results when following the provided assay protocol. Please refer to the troubleshooting guide below when unexpected difficulties are encountered. If you require further assistance, talk to a scientist at Biosensis (biospeak@biosensis.com).

Problem	Cause	Solution
High background (blank OD > 0.30)	Insufficient washing	Make sure to perform 5 washes after each incubation step with sample, detection antibody and streptavidin-HRP conjugate. Follow the recommended manual washing procedure for optimal washing performance
	Excessive concentration of detection antibody and/or HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume.
	Increased incubation time and temperature	Please follow incubation times as stated in the protocol and perform incubations at room temperature
	Contamination	Ensure that all plastic- and glassware used is clean. Do not use the same pipette tip to transfer samples and blank solution into the wells
Low absorbance readings	Concentration of Cystatin C in the sample is lower than the detection limit of this assay	Confirm that the assay protocol was carried out correctly by spiking a known amount of Cystatin C into your sample and check that the sample value falls within the expected Cystatin C concentration range
	Insufficient antibody or insufficient HRP-conjugate	Re-examine the calculations performed to dilute the concentrated stock solutions. Verify that the pipettes dispense the correct volume
	Reagents expired	Ensure correct storage of the kit and do not use the kit beyond its expiry date
	Decreased incubation times and temperature	Please follow incubation times as stated in the protocol and warm reagents to room temperature before use
	Microplate wells dried out	Do not leave microplate wells without solution for an extended time
	Wash buffer not diluted	Ensure that the wash buffer is diluted with ultrapure water to a 1x working solution
	Stop solution not added	Add the stop solution to obtain a yellow reaction product that can be measured at 450 nm
Standard OD values above plate reader limit	Excessive incubation with TMB substrate solution	Reduce incubation time by stopping the reaction at an earlier time-point
Sample OD values above standard curve range	Cystatin C concentration in sample is too high	Perform a serial dilution of your sample to obtain a reading that falls within the standard curve
High coefficient of variations (CV)	TMB and stopping solution not added consistently	Add TMB and stopping solution to the wells in the same order and speed
	Inconsistent pipetting	Ensure pipettes are working correctly and are calibrated; review your pipetting technique; add standards and samples to the wells using a single-channel rather than a multi-channel pipette
	Insufficient mixing of reagents	Briefly vortex to mix solutions before pipetting into the well
Problem	**Cause**	**Solution**