AssayMax™

Human Cystatin-S ELISA Kit

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For any questions regarding troubleshooting or performing the assay, please contact our support team at support@assaypro.com.

Thank you for choosing Assaypro.

Assay Summary

Step 1. Add 50 µl of Standard or Sample per well.
Incubate 2 hours.

Step 2. Wash, then add 50 µl of Biotinylated Antibody per well.
Incubate 1 hour.

Step 3. Wash, then add 50 µl of SP Conjugate per well.
Incubate 30 minutes.

Step 4. Wash, then add 50 µl of Chromogen Substrate per well.
Incubate 25 minutes.

Step 5. Add 50 µl of Stop Solution per well.
Read at 450 nm immediately.

Symbol Key

Consult instructions for use.

Assay Template

	A	B	C	D	E	F	G	H
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AssayMax™ Human Cystatin-S (CST4) ELISA Kit

Catalog No. EC2442-7

Sample insert for reference use only

Positive Control Included

Introduction

Cystatin-S (CST4), a cysteine proteinase inhibitor of human saliva, is a member of the type II cystatin gene family. This salivary S-type cystatin is a secreted small protein encoded by the CST4 gene and highly expressed in saliva, tears, and seminal fluid. It comprises a signal peptide of 20 amino acids, followed by a secreted peptide of 121 amino acids with about 14 kDa and two intrachain disulfide bonds (1). Cystatin-S inhibits papain and ficin strongly but does not inhibit either porcine cathepsin B or clostripain (2). As a defense protein mainly produced by submandibular glands, salivary cystatin-S is involved in innate oral immunity. Cystatin-S is significantly decreased in Sjögren's syndrome patients (3). Cystatin-S mRNA and protein expression are upregulated in gastrointestinal cancer tissues and cell lines (4). Cystatin-S is highly expressed in ovarian cancer patients and associated with poor prognoses (5).

Principle of the Assay

The AssayMax™ Human Cystatin-S ELISA (Enzyme-Linked Immunosorbent Assay) Kit is designed for detection of CST4 in human **saliva and cell culture samples**. This assay employs a quantitative **sandwich enzyme immunoassay** technique that measures human CST4 in approximately 4 hours. A polyclonal antibody specific for human CST4 has been pre-coated onto a 96-well microplate with removable strips. CST4 in standards and samples is sandwiched by the immobilized antibody and a biotinylated polyclonal antibody specific for human CST4, which is recognized by a streptavidin-peroxidase (SP) conjugate. All unbound material is washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

Caution and Warning

- This product is for **Research Use Only** and is not intended for use in diagnostic procedures.
- Prepare all reagents (diluent buffer, wash buffer, standard, biotinylated antibody, and SP conjugate), as instructed, prior to running the assay.

- Prepare all samples prior to running the assay. The dilution factors for the samples are suggested in this insert. However, the user should determine the optimal dilution factor.
- Spin down the SP conjugate vial, the biotinylated antibody vial, and the standard diluent vial before opening and using contents.
- The Stop Solution is an acidic solution.
- The kit should not be used beyond the expiration date.

Reagents

- **Human Cystatin-S Microplate:** A 96-well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against human CST4.
- **Sealing Tapes:** Each kit contains 3 precut, pressure sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **Human Cystatin-S Standard:** Human CST4 in a buffered protein base, (2000 pg, lyophilized).
- **Biotinylated Human Cystatin-S Antibody (50x):** A 50-fold concentrated biotinylated polyclonal antibody against human CST4 (120 µl).
- **EIA Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (30 ml).
- **Standard Diluent (1x):** A buffered protein base with stabilizer (2 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml, 2 bottles).
- **SP Conjugate (100x):** A 100-fold concentrate (80 µl).
- **Chromogen Substrate (1x):** A stabilized peroxidase chromogen substrate tetramethylbenzidine (7 ml).
- **Stop Solution (1x):** A 0.5 N hydrochloric acid solution to stop the chromogen substrate reaction (11 ml).
- **Positive Control:** 1 vial, lyophilized. See insert CEC24421.

Storage Condition

- Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.
- Store Standard, SP Conjugate, and Biotinylated Antibody at -20°C.
- Store Microplate, Diluent Concentrate (10x), Standard Diluent (1x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8°C.
- Unused microplate wells may be returned to the foil pouch with the desiccant packs and resealed. May be stored for up to 30 days in a vacuum desiccator.

Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm

- Pipettes (1-20 µl, 20-200 µl, 200-1000 µl, and multiple channel)
- Deionized or distilled reagent grade water

Sample Collection, Preparation, and Storage

- **Saliva:** Collect saliva using sample tube. Centrifuge samples at 800 x *g* for 10 minutes. An 80000-fold sample dilution is suggested into EIA Diluent or within the range of 5000x – 500000x; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatant:** Centrifuge cell culture media at 1500 rpm for 10 minutes at 4°C to remove debris and collect supernatant. If necessary, dilute samples into EIA Diluent; user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -80°C. Avoid repeated freeze-thaw cycles.

  Applicable samples may also include biofluids, cell culture, and tissue homogenates. If necessary, user should determine optimal dilution factor depending on application needs.

Refer to Dilution Guidelines for further instruction.

Guidelines for Dilutions of 100-fold or Greater (*for reference only; please follow the insert for specific dilution suggested)*	
100x	**10000x**
A) 4 µl sample : 396 µl buffer (100x) = 100-fold dilution *Assuming the needed volume is less than or equal to 400 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) = 10000-fold dilution *Assuming the needed volume is less than or equal to 400 µl.*
1000x	**100000x**
A) 4 µl sample : 396 µl buffer (100x) B) 24 µl of A : 216 µl buffer (10x) = 1000-fold dilution *Assuming the needed volume is less than or equal to 240 µl.*	A) 4 µl sample : 396 µl buffer (100x) B) 4 µl of A : 396 µl buffer (100x) C) 24 µl of B : 216 µl buffer (10x) = 100000-fold dilution *Assuming the needed volume is less than or equal to 240 µl.*

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **EIA Diluent Concentrate (10x):** Dilute the EIA Diluent Concentrate 10-fold with reagent grade water to produce a 1x solution. When diluting

the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved. Store for up to 30 days at 2-8°C.

- **Human Cystatin-S Standard:** Reconstitute the Human Cystatin-S Standard (2000 pg) with 0.5 ml of **Standard Diluent** to generate a 4000 pg/ml standard stock solution. Allow the vial to sit for 10 minutes with gentle agitation prior to making dilutions. From the standard stock solution (4000 pg/ml), dilute 4-fold with **EIA Diluent** to produce a 1000 pg/ml standard working solution. Prepare duplicate or triplicate standard points by serially diluting the standard working solution (1000 pg/ml) 2-fold with equal volume of EIA Diluent to produce 500, 250, 125, 62.5, 31.25, and 15.625 pg/ml solutions. EIA Diluent serves as the zero standard (0 pg/ml). Aliquot remaining stock solution to limit repeated freeze-thaw cycles. This solution should be stored at -20°C and **used within 30 days**.

Standard Point	Dilution	[CST4] (pg/ml)
P1	1 part Standard (4000 pg/ml) + 3 parts EIA Diluent	1000
P2	1 part P1 + 1 part EIA Diluent	500
P3	1 part P2 + 1 part EIA Diluent	250
P4	1 part P3 + 1 part EIA Diluent	125
P5	1 part P4 + 1 part EIA Diluent	62.5
P6	1 part P5 + 1 part EIA Diluent	31.25
P7	1 part P6 + 1 part EIA Diluent	15.625
P8	EIA Diluent	0.0

- **Biotinylated Human Cystatin-S Antibody (50x):** Spin down the antibody briefly and dilute the desired amount of the antibody 50-fold with EIA Diluent to produce a 1x solution. The undiluted antibody should be stored at -20°C.
- **Wash Buffer Concentrate (20x):** Dilute the Wash Buffer Concentrate 20-fold with reagent grade water to produce a 1x solution. When diluting the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the 1x solution gently until the crystals have completely dissolved.
- **SP Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 100-fold with EIA Diluent to produce a 1x solution. The undiluted conjugate should be stored at -20°C.

Assay Procedure

- Prepare all reagents, standard solutions, and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-25°C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl of Human Cystatin-S Standard or sample to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 2 hours. Start the timer after the last addition.
- Wash the microplate manually or automatically using a microplate washer. Invert the plate and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If washing manually, wash five times with 200 µl of Wash Buffer per well. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a microplate washer, wash six times with 300 µl of Wash Buffer per well; invert the plate and hit 4-5 times on absorbent material to completely remove the liquid.
- Add 50 µl of Biotinylated Human Cystatin-S Antibody to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 1 hour.
- Wash the microplate as described above.
- Add 50 µl of SP Conjugate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash the microplate as described above.
- Add 50 µl of Chromogen Substrate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Incubate in ambient light for 25 minutes or until the optimal blue color density develops.
- Add 50 µl of Stop Solution to each well. The color will change from blue to yellow. Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed.
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may be generated at high concentration points after stopping the reaction for about 10 minutes, which will reduce the readings.

Data Analysis

- Calculate the mean value of the duplicate or triplicate readings for each standard and sample.
- To generate a standard curve, plot the graph using the standard concentrations on the x-axis and the corresponding mean 450 nm absorbance (OD) on the y-axis. The best fit line can be determined by regression analysis using log-log or four-parameter logistic curve fit.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

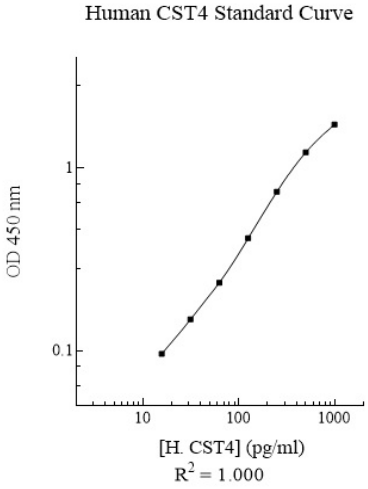
Typical Data

- The typical data is provided for reference only. Individual laboratory means may vary from the values listed. Variations between laboratories may be caused by technique differences.

Standard Point	pg/ml	OD	Average OD
P1	1000	1.760 1.682	1.721
P2	500	1.184 1.238	1.211
P3	250	0.750 0.730	0.740
P4	125	0.405 0.417	0.411
P5	62.5	0.245 0.225	0.235
P6	31.25	0.146 0.150	0.148
P7	15.625	0.100 0.092	0.096
P8	0.0	0.045 0.043	0.044
Sample: Pooled Normal Saliva (80000x)		0.386 0.374	0.380

Standard Curve

- The curve is provided for illustration only. A standard curve should be generated each time the assay is performed.



Performance Characteristics

- This assay recognizes both natural and recombinant human CST4.
- The minimum detectable dose of human CST4 as calculated by 2SD from the mean of a zero standard was established to be 7.2 pg/ml.
- Intra-assay precision was determined by testing three reference control samples twenty times in one assay.
- Inter-assay precision was determined by testing three reference control samples in twenty assays.

	Intra-Assay Precision			**Inter-Assay Precision**		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
CV (%)	5.6%	4.0%	4.5%	10.7%	9.3%	8.4%
Average CV (%)	4.7%			9.5%		

Spiking Recovery

- Recovery was determined by spiking one serum and one saliva sample with different CST4 concentrations.

Sample	Unspiked Sample (pg/ml)	Spiking Value (pg/ml)	Expected	Observed	Recovery (%)
Serum	338.007	463.059	801.066	831.048	104%
		122.862	460.869	453.160	98%
		35.710	373.717	342.793	92%
Saliva	153.639	463.059	616.698	639.002	104%
		122.862	276.501	270.999	98%
		35.710	189.349	192.484	102%
Average Recovery (%)					100%

Linearity

- Saliva samples were serially diluted to test for linearity.

Average Percentage of Expected Value (%)	
Sample Dilution	Saliva
40000x	98%
80000x	96%
160000x	105%

Cross-Reactivity

Protein	**Cross-Reactivity (%)**
CST1	10%

- No significant cross-reactivity observed with CSTA, CSTB, CST3, CST5, CST6, CST7, CST9, CST11, fetuin-A, kininogen (HMW), and kallikrein-2.

Troubleshooting

Issue	**Causes**	**Course of Action**
Low Precision	Use of improper components	• Check the expiration date listed before use. • Do not interchange components from different lots.
	Improper wash step	• Check that the correct wash buffer is being used. • Check that all wells are empty after aspiration. • Check that the microplate washer is dispensing properly. • If washing by pipette, check for proper pipetting technique.
	Splashing of reagents while loading wells	• Pipette properly in a controlled and careful manner.
	Inconsistent volumes loaded into wells	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the lyophilized components after reconstitution. • Thoroughly mix dilutions.

	Improperly sealed microplate	• Check the microplate pouch for proper sealing. • Check that the microplate pouch has no punctures. • Check that three desiccants are inside the microplate pouch prior to sealing.
Unexpectedly Low or High Signal Intensity	Microplate was left unattended between steps	• Each step of the procedure should be performed uninterrupted.
	Omission of step	• Consult the provided procedure for complete list of steps.
	Steps performed in incorrect order	• Consult the provided procedure for the correct order.
	Insufficient amount of reagents added to wells	• Check pipette calibration. • Check pipette for proper performance.
	Wash step was skipped	• Consult the provided procedure for all wash steps.
	Improper wash buffer	• Check that the correct wash buffer is being used.
	Improper reagent preparation	• Consult reagent preparation section for the correct dilutions of all reagents.
	Insufficient or prolonged incubation periods	• Consult the provided procedure for correct incubation time.
Deficient Standard Curve Fit	Non-optimal sample dilution	• Sandwich ELISA: If samples generate OD values higher than the highest standard point (P1), dilute samples further and repeat the assay. • Competitive ELISA: If samples generate OD values lower than the highest standard point (P1), dilute samples further and repeat the assay. • User should determine the optimal dilution factor for samples.
	Contamination of reagents	• A new tip must be used for each addition of different samples or reagents during the assay procedure.
	Contents of wells evaporate	• Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature.
	Improper pipetting	• Pipette properly in a controlled and careful manner. • Check pipette calibration. • Check pipette for proper performance.
	Insufficient mixing of reagent dilutions	• Thoroughly agitate the lyophilized components after reconstitution. • Thoroughly mix dilutions.

References

(1) Saitoh E *et al*. (1987) *Gene*. 6:329-338.
(2) Isemura S *et al*. (1984) *J Biochem*. 96(4):1311-1314.
(3) Martini D *et al*. (2017) *Rheumatology (Oxford)*. 56(6):1031-1038.
(4) Dou Y *et al*. (2018) *Onco Targets Ther*. 11:1743-1756.
(5) Wang S *et al*. (2021) *Life Sci*. 277:119461.