

COMPONENTS

Kit Component	Amount
96-well plate pre-coated with anti-mouse Cystatin C antibody	1 Plate
Protein Standard: Lyophilized recombinant mouse Cystatin C	2 tubes, 10 ng/tube
Sample Diluent Buffer	30 ml
Biotinylated Antibody (Anti-mouse Cystatin C)	130 µl (100x)
Antibody Diluent Buffer	12ml
Avidin-Biotin-Peroxidase Complex (ABC) Solution	130 µl (100x)
ABC Diluent Buffer	12 ml
Tetramethylbenzidine (TMB) Color Developing Agent	10 ml
TMB Stop Solution	10 ml

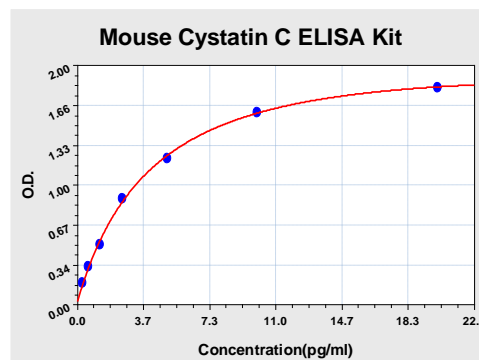
Washing Buffer (not provided): TBS or PBS

0.01M TBS: Add 1.2g Tris, 8.5g NaCl; 450µl of purified acetic acid or 700µl of concentrated hydrochloric acid to 900ml H₂O and adjust pH to 7.2-7.6. Adjust the total volume to 1L.

0.01M PBS: Add 8.5g sodium chloride, 1.4g Na₂HPO₄ and 0.2g NaH₂PO₄ to 900ml distilled H₂O and adjust pH to 7.2-7.6. Adjust the total volume to 1L.

Storage

Store at 4°C. Cell Applications, Inc. recommends using the kit within 6 months of order.



X	pg/ml	0.0	0.312	0.625	1.250	2.500	5.000	10.000	20.000
Y	O.D.450	0.030	0.187	0.325	0.513	0.895	1.227	1.611	1.817

Figure 1: Cystatin C Standard Curve. Using the Mouse Cystatin C ELISA Kit, O.D. data was graphed against Cystatin C protein concentration. The TMB reaction was incubated at 37°C for 18 min.

BACKGROUND

The cystatin protein superfamily contains three major types of inhibitors. The two human type 1 cystatins (also called stefins), cystatin A and B, are generally intracellular protease inhibitors, although low levels of these inhibitors are also found in some body fluids. The human type 2 cystatins constitute the largest group of inhibitors, comprising cystatin C, D, E/M, F, S, SA and SN, that probably have predominantly extracellular functions, as they are synthesized with signal peptides for secretion. Cystatin C shows the highest affinity and the fastest inhibition of all cystatins toward lysosomal cysteine proteases in general, and has a widespread distribution in human tissues and body fluids. Cystatin C inhibits family C1 cysteine proteases through tight and reversible binding in a substrate-competing mechanism, resulting in an equimolar complex with the protease. Human type 3 cystatins comprise L- and H-kininogen, which together with cystatin C and α2-macroglobulin are the most important plasma inhibitors of cysteine proteases.¹

Cystatin C is a non-glycosylated, basic protein (isoelectric point at pH 9.3) consisting of 120 amino acid residues. As a secreted cysteine protease inhibitor, Cystatin C has a broad spectrum of biological roles in numerous cellular systems, with growth-promoting activity, inflammation down-regulating function, and anti-viral and anti-bacterial properties as well as function in bone resorption, neutrophil chemotaxis. It is involved in numerous and varied processes such as cancer, renal diseases, diabetes, epilepsy and neurodegenerative diseases such as Alzheimer's disease (AD). It also serves as a potent inhibitor of cathepsin B and other human lysosomal cysteine proteases. Cystatin C is also known to be a better marker for renal injury than creatinine.² By inactivating cathepsin protease activity, cystatin C inhibits cancer cell invasion and metastasis. Abnormal serum levels of cystatin C or cathepsin B/cystatin C complex have been suggested as diagnostics and prognostic indicators for cancers of skin, colon and lung. Cystatin C has been suggested to play an important role in neuroendocrine differentiation of prostate cancer. More recently, serum cystatin C has been proposed as useful marker of increased osteoblastic activity associated to bisphosphonate treatments in prostate cancer patients with bone metastasis.³ In addition, it has been studied for its role in predicting new-onset or deteriorating cardiovascular disease. Cystatin C also seems to play a role in brain disorders involving amyloid (a specific type of protein deposition), such as Alzheimer's disease.⁴

References

1. Abrahamson, M. Et al: Biochem Soc Symp 70: 179-199, 2003
2. Sarkar, P.D. et al: Ind. J. Clin. Biochem. 20:139-44, 2005
3. Wegiel, B. et al: PLoS ONE 4:e7953, 2009
4. Deng, A. et al: Am. J. Path. 159:1061-1068, 2001

ELISA OVERVIEW

Cell Applications ELISA Kits are based on standard sandwich enzyme-linked immunosorbent assay technology. Freshly prepared standards, samples, and solutions are recommended for best results.

1. Prepare test samples.
2. Prepare a protein standard of the target protein.
3. Add test samples and standards to the pre-coated 96-well plate. Do not wash.
4. Add biotinylated detection antibodies. Wash.
5. Add Avidin-Biotin-Peroxidase Complex (ABC) Solution. Wash.
6. Add Tetramethylbenzidine (TMB) Color Developing Agent, containing HRP substrate.
7. Add TMB Stop Solution
8. Subject the plate to analysis.

NOTES:

- Before using the kit, quick spin tubes to bring down all solution to the bottom of tube.
- Duplicate assay wells are recommended for both standard and sample testing.
- Do not let the 96-well plate dry, this will lead to inactivation of plate components.
- When diluting samples and reagents, ensure that they are mixed completely and evenly.
- Pre-warm diluted ABC and TMB solutions at 37°C for 30 min before use to avoid variable temperature effects.
- For washes, use TBS or PBS. Do not touch well walls.
- A protein standard is included in the kit. A protein standard detection curve should be generated with each experiment, no more than 2 hours prior to the experiment.
- The user will determine sample dilution fold by estimation of target protein amount in samples.

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PROTOCOL

I. Plate Washing

Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1–2 minutes. Repeat this process two additional times for a total of three washes.

II. Preparation of Test Samples

Test Sample Processing

- **Cell culture supernate, tissue lysate or body fluids:** Remove particulates by centrifugation.
- **Serum:** Allow the serum to clot in a serum separator tube (about 2 hours) at room temperature. Centrifuge at approximately 1000 X g for 10 min.
- **Plasma:** Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 min at 1000 x g within 30 min of collection. Analyze immediately or aliquot and store frozen at -20°C.

Sample Dilution Guideline

Estimate the concentration of the target protein in the sample and select a proper dilution factor such that the diluted target protein concentration falls within the standard curve range. Depending on the sample, several trial dilutions may be necessary. Dilute the sample using the provided diluent buffer, mixing well. Suggested working dilutions of samples are as follows:

Target Protein Concentration Range	Sample Working Dilution	Sample Vol.	Diluent Buffer Vol.
200-2,000 ng/ml	1:100	1 µl	99 µl
20-200 ng/ml	1:10	10 µl	90 µl
0.312-20 ng/ml	1:2	50 µl	50 µl
≤0.312 ng/ml	n/a	100µl	n/a

If samples will be assayed within 24 hours, store at 2-8°C. For long-term storage, aliquot and freeze samples at -20°C. Avoid repeated freeze-thaw cycles.

III. Preparation of Reagents

Reconstitution of the Standard

The standard solutions should be prepared no more than 2 hours prior to the experiment. Two tubes of the standard are included in each kit. Use one tube for each experiment.

1. 20ng/ml of Mouse Cystatin C standard solution: Add 0.5 ml sample diluent buffer into one tube, keep the tube at room temperature for 10 min and mix thoroughly.
2. 10ng/ml→0.312ng/ml of Mouse Cystatin C standard solutions: Label 6 Eppendorf tubes with 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml, 0.312ng/ml, respectively. Aliquot 0.3 ml of the sample diluent buffer into each tube. Add 0.3 ml of the above 20ng/ml Cystatin C standard solution into 1st tube and mix. Transfer 0.3 ml from 1st tube to 2nd tube and mix. Transfer 0.3 ml from 2nd tube to 3rd tube and mix, and so on.

Preparation of Biotinylated Antibody Working Solution

The solution should be prepared no more than 2 hours prior to the experiment.

1. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
2. Biotinylated antibody should be diluted in 1:100 with the antibody diluent buffer and mixed thoroughly.

Preparation of the Avidin-Biotin-Peroxidase Complex (ABC) Working Solution

The solution should be prepared no more than 1 hour prior to the experiment.

1. The total volume should be: 0.1ml/well x (the number of wells). (Allowing 0.1-0.2 ml more than total volume)
2. Avidin- Biotin-Peroxidase Complex (ABC) should be diluted in 1:100 with the ABC dilution buffer and mixed thoroughly.

IV. ELISA

The ABC working solution and TMB color developing agent must be kept warm at 37°C for 30 min before use. When diluting samples and reagents, they must be mixed completely and evenly. A standard detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of target protein amount in samples.

1. Aliquot 0.1ml per well of the 20ng/ml, 10ng/ml, 5ng/ml, 2.5ng/ml, 1.25ng/ml, 0.625ng/ml, 0.312ng/ml Mouse Cystatin C standard solutions into the precoated 96-well plate. Add 0.1ml of the sample diluent buffer into the control well (Blank well). Add 0.1ml of each properly diluted sample of Mouse sera, plasma, body fluids, tissue lysates or cell culture supernatants to each empty well. See "Sample Dilution Guideline" for details. We recommend that each Mouse Cystatin C standard solution and each sample is measured in duplicate.
2. Seal the plate with the cover and incubate at 37°C for 90 min.
3. Remove the cover, discard plate content, and blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 0.1ml of biotinylated anti-Mouse Cystatin C antibody working solution into each well and incubate the plate at 37°C for 60 min.
5. Wash plate 3 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (**Plate Washing Method:** Discard the solution in the plate without touching the side walls. Blot the plate onto paper towels or other absorbent material. Soak each well with at least 0.3 ml PBS or TBS buffer for 1–2 minutes. Repeat this process two additional times for a total of THREE washes. Note: For automated washing, aspirate all wells and wash THREE times with PBS or TBS buffer, overfilling wells with PBS or TBS buffer. Blot the plate onto paper towels or other absorbent material.)
6. Add 0.1ml of prepared ABC working solution into each well and incubate the plate at 37°C for 30 min.
7. Wash plate 5 times with 0.01M TBS or 0.01M PBS, and each time let washing buffer stay in the wells for 1-2 min. Discard the washing buffer and blot the plate onto paper towels or other absorbent material. (See Step 5 for plate washing method).
8. Add 90 µl of prepared TMB color developing agent into each well and incubate plate at 37°C in dark for 15-20 min (**Note:** For reference only, the optimal incubation time should be determined by end user. And the shades of blue can be seen in the wells with the four most concentrated mouse Cystatin C standard solutions; the other wells show no obvious color).
9. Add 0.1ml of prepared TMB stop solution into each well. The color changes into yellow immediately.
10. Read the O.D. absorbance at 450nm in a microplate reader within 30 min after adding the stop solution.

V. Calculating Protein Concentration

- For all wells, determine O.D.450(Relative):

$$\text{O.D.450(Relative)} = \text{O.D.450(Reading)} - \text{O.D.450(Blank)}$$
- Plot the standard curve:
 Plot O.D.450(Relative) of each standard solution (Y) vs. the respective concentration of the standard solution (X). See **Figure 1** for a typical standard curve.
- The target protein concentration in samples can be interpolated from the standard curve. Multiply the interpolated concentration by the dilution factor to obtain the target protein concentration in the sample.

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