



Human A2M ELISA Kit

User Manual

Catalog # CEK1001

(Version 1.1A)

Sandwich Enzyme-Linked Immunosorbent Assay for Quantitative
Detection of Human A2M Concentrations in Cell Culture
Supernatants, Serum, Plasma.

For research use only. Not for diagnostic or therapeutic procedures.

I. INTRODUCTION.....	2
II. ASSAY PRINCIPLES.....	3
III. KIT COMPONENTS.....	4
IV. STORAGE AND STABILITY.....	4
V. MATERIALS REQUIRED BUT NOT PROVIDED.....	5
VI. HEALTH AND SAFETY PRECAUTIONS.....	5
VII. REAGENT PREPARATION.....	6
VIII. ASSAY PROCEDURE.....	9
IX. ASSAY PROCEDURE SUMMARY.....	11
X. TYPICAL DATA.....	12
XI. SENSITIVITY.....	12
XII. SPECIFICITY.....	12
XIII. CROSS REACTIVITY.....	13
XIV. REFERENCES.....	13
XV. TROUBLESHOOTING GUIDE.....	14
XVI. TECHNICAL SUPPORT.....	15
XVII. NOTES.....	15

I. INTRODUCTION

Alpha-2-macroglobulin, also known as A2M or CPAMD5 is a large plasma protein found in the blood. This gene is mapped to 12p13.31. Alpha-2-macroglobulin is a protease inhibitor and cytokine transporter. It inhibits many proteases, including trypsin, thrombin and collagenase. A2M is implicated in Alzheimer disease (AD) due to its ability to mediate the clearance and degradation of A-beta, the major component of beta-amyloid deposits. This gene is able to inhibit all four classes of proteinases by a unique 'trapping' mechanism. This protein has a peptide stretch, called the 'bait region' which contains specific cleavage sites for different proteinases. When a proteinase cleaves the bait region, a conformational change is induced in the protein which traps the proteinase. The entrapped enzyme remains active against low molecular weight substrates (activity against high molecular weight substrates is greatly reduced). Following cleavage in the bait region a thioester bond is hydrolyzed and mediates the covalent binding of the protein to the proteinase.

II. ASSAY PRINCIPLES

The Cohesion Bioscience Human A2M ELISA (Enzyme-Linked Immunosorbent Assay) kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of Human A2M in Cell Culture Supernatants, Serum, Plasma. This assay employs an antibody specific for Human A2M coated on a 96-well plate. Standards and samples are pipetted into the wells and A2M present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-Human A2M antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of A2M bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

III. KIT COMPONENTS

Component	Volume
96-well Plate Coated With Anti-Human A2M Antibody	8 wells x 12 Strips
Human A2M Standard	40 ng x 2
Biotin-Labeled Detection Antibody (100X)	120 µl
Streptavidin-HRP (100X)	120 µl
Standard/Sample Diluent	30 ml
Detection Antibody Diluent	12 ml
Streptavidin-HRP Diluent	12 ml
Wash Buffer (20X)	30 ml
TMB Substrate Solution	12 ml
Stop Solution	12 ml
Plate Adhesive Strips	3 Strips
Technical Manual	1 Manual

IV. STORAGE AND STABILITY

All kit components are stable at 2 to 8 °C. Standard (recombinant protein) should be stored at -20 °C or -80 °C (recommended at -80 °C) after reconstitution. Opened Microplate Wells or reagents may be store for up to 1 month at 2 to 8 °C. Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

Note: the kit can be used within one year if the whole kit is stored at -20 °C. Avoid repeated freeze-thaw cycles.

V. MATERIALS REQUIRED BUT NOT PROVIDED

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Adjustable pipettes and pipette tips to deliver 2 µl to 1 ml volumes.
3. Adjustable 1-25 ml pipettes for reagent preparation.
4. 100 ml and 1 liter graduated cylinders.
5. Absorbent paper.
6. Distilled or deionized water.
7. Computer and software for ELISA data analysis.
8. Tubes to prepare standard or sample dilutions.

VI. HEALTH AND SAFETY PRECAUTIONS

1. Reagents provided in this kit may be harmful if ingested, inhaled or absorbed through the skin. Please carefully review the MSDS for each reagent before conducting the experiment.
2. Stop Solution contains 2 N Sulfuric Acid (H_2SO_4) and is an extremely corrosive agent. Please wear proper eye, hand and face protection when handling this material. When the experiment is finished, be sure to rinse the plate with copious amounts of running water to dilute the Stop Solution prior to disposing the plate.

VII. REAGENT PREPARATION

1. Sample Preparation

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

Cell culture supernates: Remove particulates by centrifugation, assay immediately or aliquot and store samples at -20°C.

Serum: Allow the serum to clot in a serum separator tube (about 4 hours) at room temperature. Centrifuge at approximately 1000 X g for 15 minutes. Analyze the serum immediately or aliquot and store samples at -20°C.

Plasma: Collect plasma using heparin or EDTA as an anticoagulant. Centrifuge for 15 minutes at 1500 X g within 30 minutes of collection. Assay immediately or aliquot and store samples at -20°C.

Cell Lysates: Collect cells and rinse cells with PBS. Homogenize and lyse cells thoroughly in lysate solution. Centrifuge cell lysates at approximately 10000 X g for 5 minutes to remove debris. Aliquots of the cell lysates were removed and assayed.

Bone Tissue: Extract demineralized bone samples in 4 M Guanidine-HCl and protease inhibitors. Dissolve the final sample in 2 M Guanidine-HCl.

Tissue Homogenates: The preparation of tissue homogenates will vary depending upon tissue type. Rinse tissue with 1X PBS to remove excess blood, homogenized in 20 mL of 1X PBS and stored overnight at $\leq -20^{\circ}\text{C}$. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g. The supernate was removed immediately and assayed.

Alternatively, aliquot and store samples at $\leq -20^{\circ}\text{C}$.

Note: Some lysis buffer, such as RIPA can not be used. Some components will affect the binding.

Urine: Urinary samples should be cleared by centrifugation and then can be used directly without dilution. Storage at -20°C.

2. Human A2M Standard Preparation

Reconstitute the lyophilized Human A2M Standard by adding 1 ml of Standard/Sample Diluent to make the 40000 pg/ml standard stock solution. Allow solution to sit at room temperature for 5 minutes, then gently vortex to mix completely. Use within one hour of reconstituting. Two tubes of the standard (40 ng per tube) are included in each kit. Use one tube for each experiment.

Perform 2-fold serial dilutions of the top standards to make the standard curve within the range of this assay (625 pg/ml - 40000 pg/ml) as below. Standard/Sample Dilution Buffer serves as the zero standard (0 pg/ml).

Standard	Add	Into
40000 pg/ml		
20000 pg/ml	500 µl of the Standard (40000 pg/ml)	500 µl of the Standard/Sample Diluent
10000 pg/ml	500 µl of the Standard (20000 pg/ml)	500 µl of the Standard/Sample Diluent
5000 pg/ml	500 µl of the Standard (10000 pg/ml)	500 µl of the Standard/Sample Diluent
2500 pg/ml	500 µl of the Standard (5000 pg/ml)	500 µl of the Standard/Sample Diluent
1250 pg/ml	500 µl of the Standard (2500 pg/ml)	500 µl of the Standard/Sample Diluent
625 pg/ml	500 µl of the Standard (1250 pg/ml)	500 µl of the Standard/Sample Diluent
0 ng/ml	1 ml of the Standard/Sample Diluent	

Note: The standard solutions are best used within 2 hours. The 40000 pg/ml standard solution should be stored at 4°C for up to 12 hours, or at -20°C for up to 48 hours. Avoid repeated freeze-thaw cycles.

3. Biotin-Labeled Detection Antibody Working Solution Preparation

The Biotin-Labeled Detection Antibody should be diluted in 1:100 with the Detection Antibody Diluent and mixed thoroughly. The solution should be prepared no more than 2 hours prior to the experiment.

4. Streptavidin-HRP Working Solution Preparation

The Streptavidin-HRP should be diluted in 1:100 with the Streptavidin-HRP Diluent and mixed thoroughly. The solution should be prepared no more than 1 hour prior to the experiment.

5. Wash Buffer Working Solution Preparation

Pour entire contents (30 ml) of the Wash Buffer Concentrate into a clean 1,000 ml graduated cylinder. Bring final volume to 600 ml with glass-distilled or deionized water (1:20).

VIII. ASSAY PROCEDURE

The Streptavidin-HRP Working Solution and TMB Substrate Solution must be kept warm at 37°C for 30 minutes before use. When diluting samples and reagents, they must be mixed completely and evenly. Standard detection curve should be prepared for each experiment. The user will decide sample dilution fold by crude estimation of protein amount in samples.

1. Add 100 μ l of each standard and sample into appropriate wells.
2. Cover well and incubate for 90 minutes at room temperature or over night at 4°C with gentle shaking.
3. Remove the cover, discard the solution and wash plate 3 times with Wash Buffer Working Solution, and each time let Wash Buffer Working Solution stay in the wells for 1 - 2 minutes. Blot the plate onto paper towels or other absorbent material. Do NOT let the wells completely dry at any time.
4. Add 100 μ l of Biotin-Labeled Detection Antibody Working Solution into each well and incubate the plate at 37°C for 60 minutes.
5. Wash plate 3 times with Wash Buffer Working Solution, and each time let Wash Buffer Working Solution stay in the wells for 1 - 2 minutes. Discard the Wash Buffer Working Solution and blot the plate onto paper towels or other absorbent material.
6. Add 100 μ l of Streptavidin-HRP Working Solution into each well and incubate the plate at 37°C for 45 minutes.
7. Wash plate 5 times with Wash Buffer Working Solution, and each time let wash buffer stay in the wells for 1 - 2 minutes. Discard the wash buffer and blot the plate onto paper towels or other absorbent material.
8. Add 100 μ l of TMB Substrate Solution into each well and incubate plate at 37°C in dark for 10-20 minutes.
9. Add 100 μ l of 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 minutes after adding the Stop Solution.

For calculation, (the relative O.D.450) = (the O.D.450 of each well) - (the O.D.450 of Zero well). 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). The concentration of the samples can be interpolated from the standard curve.

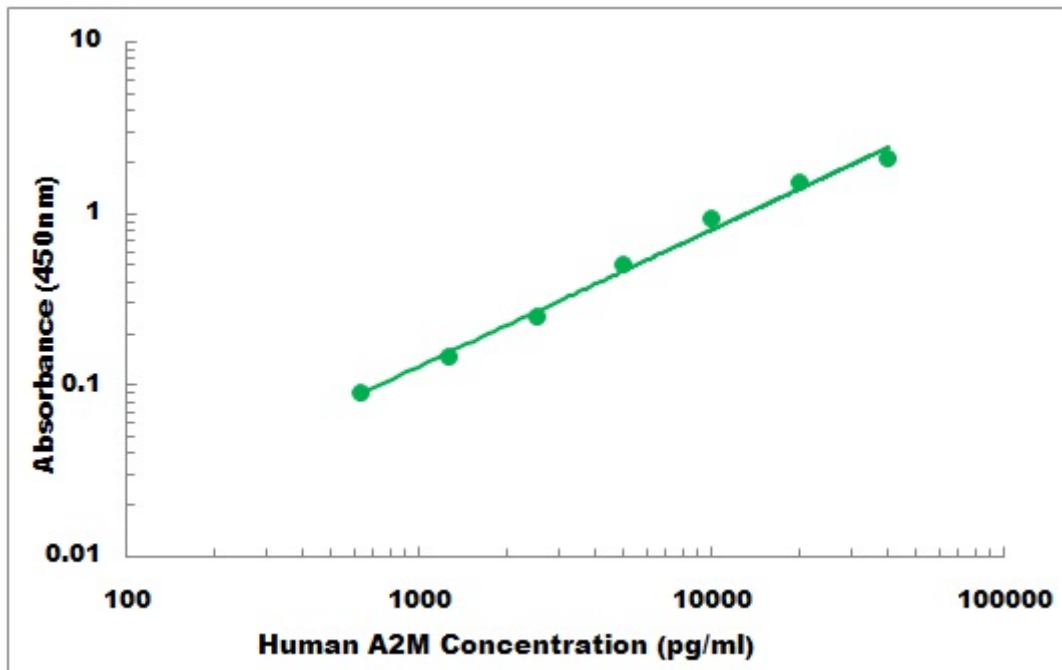
Note: If the samples measured were diluted, multiply the dilution factor to the concentrations from interpolation to obtain the concentration before dilution.

IX. ASSAY PROCEDURE SUMMARY



X. TYPICAL DATA

The standard curve is for demonstration only. A standard curve must be run with each assay.



XI. SENSITIVITY

The minimum detectable dose of Human A2M is typically less than 20 pg/ml.

XII. SPECIFICITY

The Human A2M ELISA Kit allows for the detection and quantification of endogenous levels of natural and/or recombinant Human A2M proteins within the range of 625 pg/ml - 40000 pg/ml.

XIII. CROSS REACTIVITY

No detectable cross-reactivity with other relevant proteins.

XIV. REFERENCES

1. Bian, L., Yang, J. D., Guo, T. W., Duan, Y., Qin, W., Sun, Y., Feng, G. Y., He, L.
Association study of the A2M and LRP1 genes with Alzheimer disease in the Han Chinese. *Biol. Psychiat.* 58: 731-737, 2005.
2. Flachsbart, F., Caliebe, A., Nothnagel, M., Kleindorp, R., Nikolaus, S., Schreiber, S., Nebel, A. Depletion of potential A2M risk haplotype for Alzheimer's disease in long-lived individuals. *Europ. J. Hum. Genet.* 18: 59-61, 2010.
3. Zappia, M., Manna, I., Serra, P., Cittadella, R., Andreoli, V., La Russa, A., Annesi, F., Spadafora, P., Romeo, N., Nicoletti, G., Messina, D., Gambardella, A., Quattrone, A.
Increased risk for Alzheimer disease with the interaction of MPO and A2M polymorphisms. *Arch. Neurol.* 61: 341-344, 2004.

XV. TROUBLESHOOTING GUIDE

Problem	Possible Cause	Solution
High signal and background in all wells	• Insufficient washing	• Increase number of washes • Increase time of soaking between in wash
	• Too much Streptavidin-HRP	• Check dilution, titration
	• Incubation time too long	• Reduce incubation time
	• Development time too long	• Decrease the incubation time before the stop solution is added
No signal	• Reagent added in incorrect order, or incorrectly prepared	• Review protocol
	• Standard has gone bad (If there is a signal in the sample wells)	• Check the condition of stored standard
	• Assay was conducted from an incorrect starting point	• Reagents allows to come to 20 - 30 °C before performing assay
Too much signal-whole plate turned uniformly blue	• Insufficient washing-unbound Streptavidin-HRP remaining	• Increase number of washes Carefully
	• Too much Streptavidin-HRP	• Check dilution
	• Plate sealer or reservoir reused, resulting in presence of residual Streptavidin-HRP	• Use fresh plate sealer and reagent reservoir for each step
Standard curve achieved but poor discrimination between point	• Plate not developed long enough	• Increase substrate solution incubation time
	• Improper calculation of standard curve dilution	• Check dilution, make new standard curve
No signal when a signal is expected, but standard curve looks fine	• Sample matrix is masking detection	• More diluted sample Recommended
Samples are reading too high, but standard curve is fine	• Samples contain protein levels above assay range	• Dilute samples and run Again
Edge effect	• Uneven temperature around work surface	• Avoid incubating plate in areas where environmental conditions vary • Use plate sealer

XVI. TECHNICAL SUPPORT

For troubleshooting, information or assistance, please go online to www.cohesionbio.com or contact us at techsupport@cohesionbio.com

COHESION BIOSCIENCES LIMITED

FLAT32 ADVENTURES COURT

12 NEWPORT AVENUE

LONDON, E14 2DN, UK

Website: www.cohesionbio.com

Email: order@cohesionbio.com

techsupport@cohesionbio.com

custom@cohesionbio.com

XVII. NOTES