



AssayMax Human Antithrombin III ELISA Kit

Catalog # EA3301-1

Introduction

The serine-protease-inhibitor antithrombin III (AT III), the most important natural inhibitor of thrombin activity, has been shown to exert marked anti-inflammatory properties and proven to be efficacious in experimental models of sepsis, septic shock, and disseminated intravascular coagulation (1). It has often been recommended for the therapy of septic patients as it provides anticoagulant and anti-inflammatory actions (2). Antithrombin III (AT III) deficiency is a rare hereditary disease that predisposes to thromboembolic complications (3). AT III levels are positively correlated with plasma total cholesterol levels, plasma low-density lipoprotein cholesterol levels, plasma triglycerides and D-dimer levels (4).

Principal of the Assay

The AssayMax AT III ELISA kit is designed for detection of human AT III in urine and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique, which measures AT III in 4 hours. A monoclonal antibody specific for AT III has been pre-coated onto a microplate. AT III in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for AT III, which is recognized by a streptavidin-peroxidase conjugate. All unbound material is then 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 kit is for research use only.
- The kit should not be used beyond the expiration date.
- The Stop Solution is an acid solution.

Reagents

- **AT III Microplate:** A 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against human AT III.
- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes, which can be cut to fit the format of the individual assay.
- **AT III Standard:** Recombinant human AT III in a buffered protein base (400 ng, lyophilized).
- **Biotinylated AT III Antibody (100x):** A 100-fold concentrated biotinylated polyclonal antibody against AT III (80 µl).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (90 µl).
- **MIX Diluent Concentrate (10x):** A 10-fold concentrated buffered protein base (30 ml).
- **Wash Buffer Concentrate (20x):** A 20-fold concentrated buffered surfactant (30 ml).
- **Chromogen Substrate:** A ready-to-use stabilized peroxidase chromogen substrate tetramethylbenzidine (8 ml).

- **Stop Solution:** A 0.5 N hydrochloric acid to stop the chromogen substrate reaction (12 ml).

Storage Condition

- Store unopened kit at 2 - 8⁰C up to expiration date.
- Opened MIX Diluent may be stored for up to 1 month at 2 - 8⁰C. Store reconstituted reagents at <-20⁰C.
- Opened unused strip wells may return to the foil pouch with the desiccant pack, reseal along zip-seal. May be stored for up to 1 month 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 and Storage

- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20⁰C or below. Avoid repeated freeze-thaw cycles.
- **Urine:** Collect urine using sample pot. Centrifuge samples at 800 x g for 10 minutes and assay. Dilute samples 1:2 into MIX Diluent. Store samples at -20⁰C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.
- **MIX Diluent Concentrate (10x):** Dilute the MIX Diluent Concentrate 1:10 with reagent grade water. Store for up to 1 month at 2 - 8⁰C.
- **AT III Standard:** Reconstitute the 400 ng of human AT III Standard with 2 ml of MIX Diluent to generate a stock solution of 200 ng/ml. Allow the standard to sit for 10 minutes with gentle agitation prior to making dilutions. Prepare triplicate standard points by serially diluting the Standard solution (200 ng/ml) twofold with equal volume of MIX Diluent to produce 100, 50, 25, 12.5, 6.25, and 3.13 ng/ml. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20⁰C.

Standard Point	Dilution	[AT III] (ng/ml)
P1	1 part Standard (200 ng/ml)	200.00
P2	1 part P1 + 1 part MIX Diluent	100.00
P3	1 part P2 + 1 part MIX Diluent	50.00
P4	1 part P3 + 1 part MIX Diluent	25.00
P5	1 part P4 + 1 part MIX Diluent	12.50
P6	1 part P5 + 1 part MIX Diluent	6.25
P7	1 part P6 + 1 part MIX Diluent	3.13
P8	MIX Diluent	0.00

- **Biotinylated AT III Antibody (100x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:100 with MIX Diluent. Any remaining solution should be frozen at -20⁰C.

- **Wash Buffer Concentrate (20x):** Dilute the Wash Buffer Concentrate 1: 20 with reagent grade water.
- **Streptavidin-Peroxidase Conjugate (100x):** Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with MIX Diluent. Any remaining solution should be frozen at -20°C .

Assay Procedure

- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature ($20\text{-}30^{\circ}\text{C}$).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add $50\ \mu\text{l}$ of Standard or sample per well, and cover wells and incubate for two hours. Start the timer after the last sample addition.
- Wash five times with $200\ \mu\text{l}$ of Wash Buffer. Invert the plate and decant the contents, and blot it on absorbent paper towel to completely remove liquid at each step.
- Add $50\ \mu\text{l}$ of Biotinylated AT III Antibody to each well and incubate for one hour.
- Wash five times with $200\ \mu\text{l}$ of Wash Buffer.
- Add $50\ \mu\text{l}$ of Streptavidin-Peroxidase Conjugate per well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.
- Wash five times with $200\ \mu\text{l}$ of Wash Buffer.
- Add $50\ \mu\text{l}$ of Chromogen Substrate per well and incubate for about 10 minutes or till the optimal blue color density develops. Gently tap plate to ensure thorough mixing and break the bubbles in the well with pipette tip.
- Add $50\ \mu\text{l}$ of Stop Solution to each well. The color will change from blue to yellow.
- Read the absorbance on a microplate reader at a wavelength of 450 nm immediately.

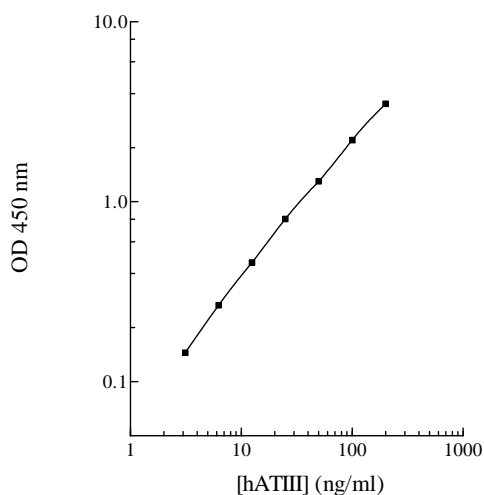
Data Analysis

- Calculate the mean value of the 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 on the y-axis and draw a best-fit curve through the points on the graph. Plotting the log-log graph may linearize the data and the best-fit line can be determined by regression analysis of the linear portion of the curve.
- Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

Standard Curve

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

Human ATIII Standard Curve



Performance Characteristics

- The minimum detectable dose of AT III is typically 2 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.8 % and 7.2% respectively.

Linearity

Sample Dilution	Average Percentage of Expected Value	
	Cell Culture Supernatant	Uine
1:4000	96%	100%
1:8000	100%	101%
1:16000	102%	102%

Recovery

Standard Added Value	5 – 50 ng/ml
Recovery %	86-112 %
Average Recovery %	99 %

Cross-Reactivity

Species	% Cross Reactivity
Beagle	None
Monkey	> 60 (suggest 1:2000 dilution for plasma)
Mouse	None
Rat	None
Swine	None

References

- (1) Oelschläger C *et. al.* (2002) *Blood* 99(11):4015-20
- (2) Kulka PJ *et. al.* (2001) *Anesthesiol Intensivmed Notfallmed Schmerzther.* 36(3): 143-53
- (3) Takahashi J. *et.al.* (2003) *Ann Thorac Cardiovasc Surg*
- (4) Erem C *et. al.* (2005) *Med Princ Pract.* 14(1): 22-30

Version 3.8

Related Products

- EA3302-1 AssayMax Human ATIII ELISA Kit (for plasma and serum samples)