



# AssayMax Mouse Antithrombin III ELISA Kit

Catalog No. EMA3301-1

## 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 Mouse AT III ELISA kit is designed for detection of mouse AT III in plasma, serum and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique, which measures AT III in less than 4 hours. A polyclonal antibody specific for mouse AT III has been pre-coated onto a microplate. Mouse AT III in standards and samples is sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for mouse 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

- **Mouse AT III Microplate:** A 96 well polystyrene microplate (12 strips of 8 wells) coated with a polyclonal antibody against mouse 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.
- **Mouse AT III Standard:** Mouse AT III in a buffered protein base (800 ng, lyophilized).
- **Biotinylated AT III Antibody (50x):** A 50-fold concentrated biotinylated polyclonal antibody against AT III (160  $\mu$ l).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (90  $\mu$ l).
- **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 kit at 2-8<sup>0</sup>C or -20<sup>0</sup>C upon arrival up to the expiration date.
- Opened MIX Diluent may be stored for up to 1 month at 2 - 8<sup>0</sup>C. Store reconstituted reagents at <-20<sup>0</sup>C.
- 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

- **Plasma:** Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 2000 x g for 10 minutes and assay. Dilute samples 1:16000 into MIX Diluent. Store samples at -20<sup>0</sup>C or below for up to 3 months. Avoid repeated freeze-thaw cycles. (EDTA or Heparin can also be used as anticoagulant.)
- **Serum:** Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 2000 x g for 10 minutes. Remove serum and assay. Dilute samples 1:16000 into MIX Diluent. Store serum at -20<sup>0</sup>C or below. Avoid repeated freeze-thaw cycles.
- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay. Store samples at -20<sup>0</sup>C or below. 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 - 8<sup>0</sup>C.
- **AT III Standard:** Reconstitute the 800 ng of Mouse AT III Standard with 2 ml of MIX Diluent to generate a stock solution of 400 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 (400 ng/ml) 1:4 with MIX Diluent to produce 100, 25, 6.25 and 1.56 ng/ml. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20<sup>0</sup>C.

Standard Point	Dilution	[mAT III] (ng/ml)
P1	1 part Standard (400 ng/ml)	400.00
P2	1 part P1 + 3 part MIX Diluent	100.00
P3	1 part P2 + 3 part MIX Diluent	25.00
P4	1 part P3 + 3 part MIX Diluent	6.25
P5	1 part P4 + 3 part MIX Diluent	1.56
P6	MIX Diluent	0.00

- **Biotinylated AT III Antibody (50x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:50 with MIX Diluent. Any remaining solution should be frozen at -20°C.
- **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

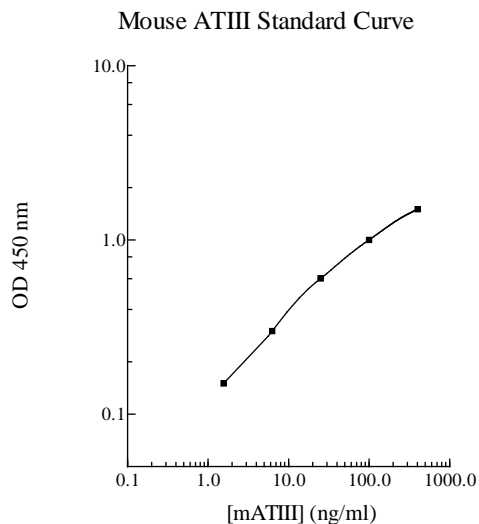
- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30°C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.
- Add 50 µl 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 µl 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 µl of Biotinylated AT III Antibody to each well and incubate for one hour.
- Wash five times with 200 µl of Wash Buffer.
- Add 50 µl 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 µl of Wash Buffer.
- Add 50 µl of Chromogen Substrate per well and incubate for about 15 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 µl 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.



## Performance Characteristics

- The minimum detectable dose of AT III is typically 1 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.7 % and 7.5% respectively.
- This kit is specific for mouse ATIII. It has less than 5 % cross-reactivity with human ATIII or rat ATIII.

## Linearity

Sample Dilution	Average Percentage of Expected Value	
	Plasma	Serum
1:8000	103%	107%
1:16000	100%	101%
1:32000	99%	97%

## Recovery

Standard Added Value	10 – 100 ng/ml
Recovery %	88-112 %
Average Recovery %	99 %

## Cross-Reactivity

Species	% Cross Reactivity
Beagle	None
Human	< 5
Monkey	< 5
Bovine	None
Rat	<5
Swine	None
Rabbit	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 1.3

## Related Products

- EA3302-1 AssayMax Human ATIII ELISA Kit (Plasma and Serum samples)
- EA3301-1 AssayMax Human ATIII ELISA Kit (Urine and Cell Culture Supernatant samples)
- ERA3301-1 AssayMax Rat ATIII ELISA Kit