Mouse Antithrombin III ELISA Kit

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). AT III deficiency is a rare hereditary disease that predisposes to throm-boembolic 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 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 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

- **Prepare all reagents (working diluent buffer, wash buffer, standards, 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 protocol. However, the user should determine the optimal dilution factor.**
- **Spin down the SP conjugate vial and the biotinylated-antibody vial before opening and using contents.**
- 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 (400 ng, lyophilized).
- **Biotinylated AT III Antibody (80x):** A 80-fold concentrated biotinylated polyclonal antibody against AT III (100 µl).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80 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, 2 bottles).
- **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 components of the kit at 2-8^{0}C or -20^{0}C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20^{0}C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8^{0}C
- Opened unused microplate wells may be returned to the foil pouch with the desiccant packs. Reseal along zip-seal. May be stored for up to 1 month in a vacuum desiccator.
- Diluent (1x) may be stored for up to 1 month at 2-8^{0}C.
- Store Standard at 2-8^{0}C before reconstituting with Diluent and at -20^{0}C after reconstituting with Diluent.

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^{0}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^{0}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^{0}C or below. Avoid repeated freeze-thaw cycles.

Reagent Preparation

- Freshly dilute all reagents and bring all reagents to room temperature before use.
- **MIX Diluent Concentrate (10x)**: If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent Concentrate 1:10 with reagent grade water. Store for up to 1 month at 2 - 8^{0}C.
- **AT III Standard:** Reconstitute the 400 ng of Mouse AT III Standard with 1 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 duplicate or triplicate standard points by serially diluting the Standard solution (400 ng/ml) 1:4 with MIX Diluent to produce 100, 25, 6.25, 1.563 and 0.391 ng/ml. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20^{0}C.

Standard Point	Dilution	[mAT III] (ng/ml)
P1	1 part Standard (400 ng/ml) + 3 parts MIX Diluent	100.00
P2	1 part P1 + 3 parts MIX Diluent	25.00
P3	1 part P2 + 3 parts MIX Diluent	6.250
P4	1 part P3 + 3 parts MIX Diluent	1.563
P5	1 part P4 + 3 parts MIX Diluent	0.391
P6	MIX Diluent	0.000

- **Biotinylated AT III Antibody (80x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:80 with MIX Diluent. Any remaining solution should be frozen at -20^{0}C.
- **Wash Buffer Concentrate (20x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved 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^{0}C.

Assay Procedure

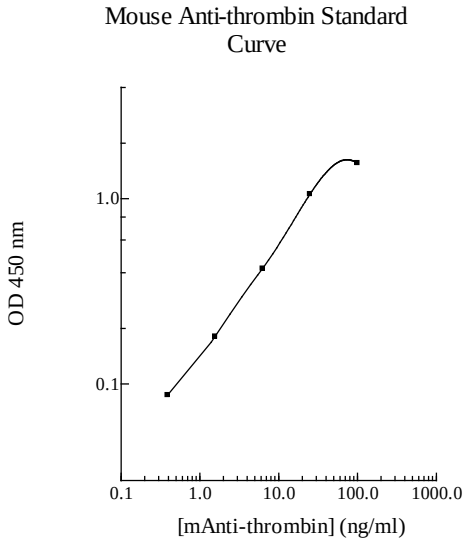
- Prepare all reagents, working standards and samples as instructed. Bring all reagents to room temperature before use. The assay is performed at room temperature (20-30^{0}C).
- Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccant inside. 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 manually. Invert the plate each time and decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
  If using a machine wash six times with 300 µl of Wash Buffer and then invert the plate, decant the contents; hit it 4-5 times on absorbent paper towel to completely remove the liquid.
- Add 50 µl of Biotinylated AT III Antibody to each well and incubate for one hour.
- Wash the microplate as described above.
- 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 the microplate as described above.
- Add 50 µl of Chromogen Substrate per well and incubate for about 30 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**. 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 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 ~0.4 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.8 % and 7.6% respectively.
- This kit is specific for mouse ATIII. It has less than 5 % cross-reactivity with human ATIII or rat ATIII.

Linearity

	Average Percentage of Expected Value	
Sample Dilution	**Plasma**	**Serum**
1:8000	91%	95%
1:16000	100%	101%
1:32000	102%	103%

Recovery

Standard Added Value	2 – 20 ng/ml
Recovery %	87-111 %
Average Recovery %	97 %

Cross-Reactivity

Species	% Cross Reactivity
Canine	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) *Anasthesiol Intensivmed Notfallmed Schmerzther*. 36(3): 143-53.
(3) Takahashi J. *et.al.* (2003) *Ann Thorac Cardiovasc Surg.* 9(3):192-6.
(4) Erem C *et al.* (2005) *Med Princ Pract.* 14(1): 22-30

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