

## **Human Thrombin-antithrombin (TAT) Complexes ELISA Kit**

### **Introduction**

Thrombin-antithrombin (TAT) complexes formed following the neutralization of thrombin by antithrombin III (ATIII) have been used as a surrogate marker for thrombin generation (1). High plasma level of TAT complexes has been suggested to alter hemostatic activation in argentine hemorrhagic fever (2), chronic dialysis patients (3), and toxemia of pregnancy (4). Whereas low plasma level of TAT complexes is found in type 1 (insulin-dependent) diabetes (5), neonatal respiratory distress syndrome (6), and primary untreated cancer (7). TAT complexes are a useful marker to predict morphological changes in chronic aortic dissection (8).

### **Principal of the Assay**

The TAT complexes ELISA kit is designed for detection of human TAT complexes in plasma, milk, and cell culture supernatants. This assay employs a quantitative sandwich enzyme immunoassay technique that measures TAT complexes in less than 4 hours. A monoclonal antibody specific for Antithrombin has been pre-coated onto a microplate. TAT complexes in standards and samples are sandwiched by the immobilized antibody and biotinylated polyclonal antibody specific for Thrombin, 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**

- **Antithrombin Microplate:** A 96 well polystyrene microplate (12 strips of 8 wells) coated with a monoclonal antibody against Antithrombin.

- **Sealing Tapes:** Each kit contains 3 pre-cut, pressure-sensitive sealing tapes that can be cut to fit the format of the individual assay.
- **TAT Complexes Standard:** Human TAT complexes in a buffered protein base (240 ng, lyophilized).
- **Biotinylated Thrombin Antibody (30x):** A 30-fold concentrated biotinylated polyclonal antibody against thrombin (270  $\mu$ 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, 2 bottles).
- **Streptavidin-Peroxidase Conjugate (SP Conjugate):** A 100-fold concentrate (80  $\mu$ l).
- **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<sup>0</sup>C or -20<sup>0</sup>C upon arrival up to the expiration date.
- Store SP Conjugate and Biotinylated Antibody at -20<sup>0</sup>C
- Store Microplate, Diluent Concentrate (10x), Wash Buffer, Stop Solution, and Chromogen Substrate at 2-8<sup>0</sup>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<sup>0</sup>C.
- Store Standard at 2-8<sup>0</sup>C before reconstituting with Diluent and at -20<sup>0</sup>C after reconstituting with Diluent.

## Other Supplies Required

- Microplate reader capable of measuring absorbance at 450 nm.
- Pipettes (1-20  $\mu$ l, 20-200  $\mu$ l, 200-1000  $\mu$ l 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. The undiluted samples can be stored at -20<sup>0</sup>C or below for up to 1 month. Avoid repeated freeze-thaw cycles. (EDTA or Heparin can also be used as anticoagulant.)
- **Cell Culture Supernatants:** Centrifuge cell culture media at 2000 x g for 10 minutes to remove debris. Collect supernatants and assay. Samples can be stored at -20<sup>0</sup>C or below for up to 1 month. Avoid repeated freeze-thaw cycles.
- **Milk:** Collect milk using sample tube. Centrifuge samples at 800 x g for 10 minutes and assay. The undiluted samples can be stored at -20<sup>0</sup>C 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.

- **MIX Diluent Concentrate (10x):** If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the MIX Diluent 1:10 with reagent grade water. Store for up to 1 month at 2-8°C.
- **TAT Complexes Standard:** Reconstitute the 240 ng of human TAT Complexes Standard with 2 ml of MIX Diluent to generate a standard solution of 120 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 1:3 with MIX Diluent to produce 40, 13.33, 4.44, and 1.48 ng/ml. MIX Diluent serves as the zero standard (0 ng/ml). Any remaining solution should be frozen at -20°C.

Standard Point	Dilution	[TAT] (ng/ml)
P1	1 part Standard Solution (120 ng/ml)	120.00
P2	1 part P1 + 2 parts MIX Diluent	40.00
P3	1 part P2 + 2 parts MIX Diluent	13.33
P4	1 part P3 + 2 parts MIX Diluent	4.44
P5	1 part P4 + 2 parts MIX Diluent	1.48
P6	MIX Diluent	0.00

- **Biotinylated Thrombin Antibody (30x):** Spin down the antibody briefly and dilute the desired amount of the antibody 1:30 with MIX Diluent. Any remaining solution should be frozen at -20°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.
- **SP 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. Cover wells with a sealing tape 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 TAT 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 15 minutes or till the optimal color density develops. Gently tap the 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.

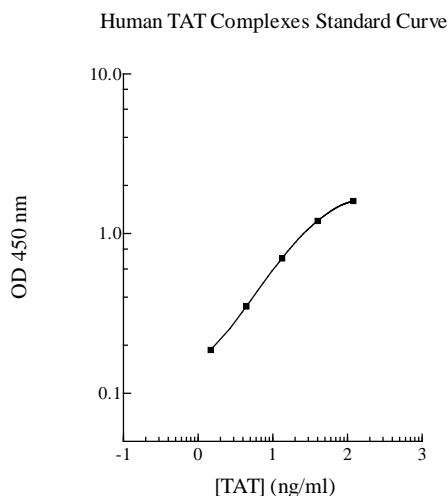
- Read the absorbance on a microplate reader at a wavelength of 450 nm **immediately**. If wavelength correction is available, subtract readings at 570 nm from those at 450 nm to correct optical imperfections. Otherwise, read the plate at 450 nm only. Please note that some unstable black particles may 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. The best-fit line can be determined by regression analysis using four-parameter or log-log logistic curve-fit.
- 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.



## Precision, Sensitivity and Specificity

- The minimum detectable dose of TAT complexes is typically ~1.5 ng/ml.
- Intra-assay and inter-assay coefficients of variation were 4.8 % and 7.3 % respectively.

## Linearity

	Average Percentage of Expected Value
Sample Dilution	Plasma
No dilution	100%
1:2	102%
1:4	104%

	Average Percentage of Expected Value
Sample Dilution	Milk
No dilution	99%
1:2	95%
1:4	96%

### Standard Added Value

Standard Added Value	2 - 20 ng/ml
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### Cross-Reactivity

Species	% Cross Reactivity
Beagle	50%
Bovine	None
Monkey	10%
Mouse	10%
Rat	10%
Swine	None
Rabbit	None
Human	100%

### References

- (1) Diquelou A *et al.* (1994) *Blood* 84(7): 2206-13
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- (3) Kario K *et al.* (1992) *Thromb Res.* 67(1): 105-13
- (4) Terao T *et al.* (1991) *Gynecol Obstet Invest.* 31(2): 74-85
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- (6) Schmidt B *et al.* (1992) *Am Rev Respir Dis.* 145(4 Pt 1): 767-70
- (7) Nanninga PB *et al.* (1990) *Thromb Haemost.* 64(3): 361-4
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Version 7.7R