

# **ab108801 – Human Antithrombin III ELISA Kit**

## Instructions for Use

For the quantitative measurement of Human Antithrombin III in urine, milk, saliva, cerebrospinal fluid, plasma, and serum.

This product is for research use only and is not intended for diagnostic use.

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## 1. BACKGROUND

Abcam's Human Antithrombin III *in vitro* ELISA (Enzyme-Linked Immunosorbent Assay) kit is designed for the quantitative measurement of Antithrombin III in urine, milk, saliva, cerebrospinal fluid, plasma, and serum.

An antithrombin III specific antibody has been precoated onto 96-well plates and blocked. Standards or test samples are added to the wells and subsequently an antithrombin III specific biotinylated detection antibody is added and then followed by washing with wash buffer. Streptavidin-Peroxidase Complex is added and unbound conjugates are washed away with wash buffer. TMB is then used to visualize Streptavidin-Peroxidase enzymatic reaction. TMB is catalyzed by Streptavidin-Peroxidase to produce a blue color product that changes into yellow after adding acidic stop solution. The density of yellow coloration is directly proportional to the amount of antithrombin III captured in plate.

The serine protease inhibitor Antithrombin 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. It has often been recommended for the therapy of septic patients as it provides anticoagulant and anti-inflammatory actions. Antithrombin III deficiency is a rare hereditary disease that predisposes to thrombo-embolic complications. Antithrombin III levels are positively correlated with plasma total cholesterol levels, plasma low-density lipoprotein cholesterol levels, plasma triglycerides and D-dimer levels.

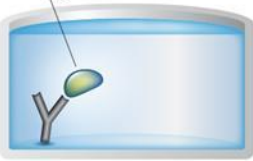
## 2. ASSAY SUMMARY

### Primary capture antibody



Prepare all reagents, samples and standards as instructed.

### Sample



Add standard or sample to each well used. Incubate at room temperature.

### Primary detector antibody



Wash and add prepared biotin antibody to each well. Incubate at room temperature.

### Streptavidin Label



Wash and add prepared Streptavidin-Peroxidase Conjugate. Incubate at room temperature.

### Substrate **Colored product**



Add Chromogen Substrate to each well. Incubate at room temperature. Add Stop Solution to each well. Read immediately.

## 3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

Modifications to the kit components or procedures may result in loss of performance.

## 4. STORAGE AND STABILITY

Store kit at 4°C immediately upon receipt, apart from the SP Conjugate & Biotinylated Antibody, which should be stored at -20°C.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in sections 9 & 10.

## 5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)
Antithrombin III Microplate (12 x 8 well strips)	96 wells	4°C
Antithrombin III Standard	1 vial	4°C
10X Diluent N Concentrate	30 mL	4°C
Biotinylated Human Antithrombin III Antibody	1 vial	-20°C
100X Streptavidin-Peroxidase Conjugate (SP Conjugate)	80 µL	-20°C
Chromogen Substrate	7 mL	4°C
Stop Solution	11 mL	4°C
20X Wash Buffer Concentrate	2 x 30 mL	4°C
Sealing Tapes	3	N/A

### **6. MATERIALS REQUIRED, NOT SUPPLIED**

These materials are not included in the kit, but will be required to successfully utilize this assay:

- 1 Microplate reader capable of measuring absorbance at 450 nm.
- Precision pipettes to deliver 1  $\mu$ L to 1 mL volumes.
- Adjustable 1-25 mL pipettes for reagent preparation.
- 100 mL and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- Log-log graph paper or computer and software for ELISA data analysis.
- 8 tubes to prepare standard or sample dilutions.

### **7. LIMITATIONS**

- Do not mix or substitute reagents or materials from other kit lots or vendors.

### 8. TECHNICAL HINTS

- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Complete removal of all solutions and buffers during wash steps.
- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**

## 9. REAGENT PREPARATION

Equilibrate all reagents to room temperature (18-25°C) prior to use. Prepare fresh reagents immediately prior to use. If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved.

### 9.1 1X Diluent N

Dilute the 10X Diluent N Concentrate 1:10 with reagent grade water. Mix gently and thoroughly. *Store for up to 1 month at 4°C.*

### 9.2 1X Wash Buffer

Dilute the 20X Wash Buffer Concentrate 1:20 with reagent grade water. Mix gently and thoroughly.

### 9.3 1X Biotinylated Antithrombin III Detector Antibody

9.3.1 The Stock Biotinylated Antithrombin III Antibody must be diluted with 1X Diluent N according to the label concentration to prepare 1X Biotinylated Antithrombin III Detector Antibody for use in the assay procedure. Observe the label for the “X” concentration on the vial of Biotinylated Antithrombin III Antibody.

9.3.2 Calculate the necessary amount of 1X Diluent N to dilute the Biotinylated Antithrombin III Antibody to prepare a 1X Biotinylated Antithrombin III Detector Antibody solution for use in the assay procedure according to how many wells you wish to use and the following calculation:

Number of Wells Strips	Number of Wells	(V <sub>T</sub> ) Total Volume of 1X Biotinylated Detector Antibody (μL)
4	32	1,760
6	48	2,640
8	64	3,520
10	80	4,400
12	96	5,280

*Any remaining solution should be frozen at -20°C.*

## ASSAY PREPARATION

### Where:

$C_S$  = Starting concentration (X) of stock Biotinylated Antithrombin III Antibody (variable)

$C_F$  = Final concentration (always = 1X) of 1X Biotinylated Antithrombin III Detector Antibody solution for the assay procedure

$V_T$  = Total required volume of 1X Biotinylated Antithrombin III Detector Antibody solution for the assay procedure

$V_A$  = Total volume of (X) stock Biotinylated Antithrombin III Antibody

$V_D$  = Total volume of 1X Diluent N required to dilute (X) stock Biotinylated Antithrombin III Antibody to prepare 1X Biotinylated Detector Antibody solution for assay procedures

Calculate the volume of (X) stock Biotinylated Antibody required for the given number of desired wells:

$$(C_F / C_S) \times V_T = V_A$$

Calculate the final volume of 1X Diluent N required to prepare the 1X Biotinylated Antithrombin III Detector Antibody:

$$V_T - V_A = V_D$$

### Example:

**NOTE: This example is for demonstration purposes only. Please remember to check your antibody vial for the actual concentration of antibody provided.**

$C_S$  = 50X Biotinylated Antithrombin III Antibody stock

$C_F$  = 1X Biotinylated Antithrombin III Detector Antibody solution for use in the assay procedure

$V_T$  = 3,520  $\mu$ L (8 well strips or 64 wells)

$$(1X/50X) \times 3,520 \mu\text{L} = 70.4 \mu\text{L}$$

$$3,520 \mu\text{L} - 70.4 \mu\text{L} = 3,449.6 \mu\text{L}$$

$V_A$  = 70.4  $\mu$ L total volume of (X) stock Biotinylated Antithrombin III Antibody required

$V_D = 3,449.6 \mu\text{L}$  total volume of 1X Diluent N required to dilute the 50X stock Biotinylated Antibody to prepare 1X Biotinylated Antithrombin III Detector Antibody solution for assay procedures

9.3.3 First spin the Biotinylated Antithrombin III Antibody vial to collect the contents at the bottom.

9.3.4 Add calculated amount  $V_A$  of stock Biotinylated Antithrombin III Antibody to the calculated amount  $V_D$  of 1X Diluent N. Mix gently and thoroughly.

#### 9.4 **1X SP Conjugate**

Spin down the 100X Streptavidin-Peroxidase Conjugate (SP Conjugate) briefly and dilute the desired amount of the conjugate 1:100 with 1X Diluent N.

*Any remaining solution should be frozen at  $-20^\circ\text{C}$ .*

## 10. STANDARD PREPARATIONS

- Prepare serially diluted standards immediately prior to use. Always prepare a fresh set of standards for every use.
- Any remaining standard should be stored at -20°C after reconstitution and used within 30 days.
- This procedure prepares sufficient standard dilutions for duplicate wells.

10.1 Reconstitution of the Antithrombin III Standard vial to prepare a 100 ng/mL Antithrombin III **Standard**:

10.1.1 First consult the Antithrombin III Standard vial to determine the mass of protein in the vial.

10.1.2 Calculate the appropriate volume of 1X Diluent N to add when resuspending the Antithrombin III Standard vial to produce a 100 ng/mL Antithrombin III **Standard** by using the following equation:

$C_S$  = Starting mass of Antithrombin III Standard (see vial label) (µg)

$C_F$  = 100 ng/mL Antithrombin III **Standard** final required concentration

$V_D$  = Required volume of 1X Diluent N for reconstitution (µL)

Calculate total required volume 1X Diluent N for resuspension:

$$(C_S / C_F) \times 1,000 = V_D$$

Example:

**NOTE: This example is for demonstration purposes only. Please remember to check your standard vial for the actual amount of standard provided.**

$C_S$  = 90 ng of Antithrombin III Standard in vial

$C_F$  = 100 ng/mL Antithrombin III **Stock Standard** final concentration

$V_D$  = Required volume of 1X Diluent N for reconstitution

$$(90 \text{ ng} / 100 \text{ ng/mL}) \times 1,000 = 900 \text{ }\mu\text{L}$$

- 10.1.3 First briefly spin the Antithrombin III Standard Vial to collect the contents on the bottom of the tube.
- 10.1.4 Reconstitute the Antithrombin III Standard vial by adding the appropriate calculated amount  $V_D$  of 1X Diluent N to the vial to generate the 100 ng/mL Antithrombin III **Standard**. Mix gently and thoroughly.
- 10.2 Allow the reconstituted 100 ng/mL Antithrombin III **Standard** to sit for 10 minutes with gentle agitation prior to making subsequent dilutions.
- 10.3 Label eight tubes #1-8.
- 10.4 Prepare the 100 ng/mL **Standard #1** by adding 240  $\mu\text{L}$  of the reconstituted 100 ng/mL Antithrombin III **Standard** to tube #1.
- 10.5 Add 120  $\mu\text{L}$  of 1X Diluent N to tubes #2 – 8.
- 10.6 To prepare **Standard #2**, add 120  $\mu\text{L}$  of the **Standard #1** into tube #2 and mix gently.
- 10.7 To prepare **Standard #3**, add 120  $\mu\text{L}$  of the **Standard #2** into tube #3 and mix gently.
- 10.8 Using the table below as a guide, prepare subsequent serial dilutions.
- 10.9 1X Diluent N serves as the zero standard, 0 ng/mL (tube #8).

# ASSAY PREPARATION

**Standard Dilution Preparation Table**

Standard #	Volume to Dilute (μL)	Volume Diluent N (μL)	Total Volume (μL)	Starting Conc. (ng/mL)	Final Conc. (ng/mL)	Final Conc. (mIU/mL)
1	Step 10.4				100.0	0.67
2	120	120	240	100.0	50.0	0.335
3	120	120	240	50.0	25.00	0.168
4	120	120	240	25.00	12.50	0.084
5	120	120	240	12.50	6.250	0.042
6	120	120	240	6.250	3.125	0.021
7	120	120	240	3.125	1.563	0.010
8	-	120	120	-	0	0



## 11. SAMPLE PREPARATION

### 11.1 **Urine:**

Collect urine using sample pot. Centrifuge samples at 800 x g for 10 minutes. Dilute samples 1:2 into 1X Diluent N or within the range of 1x – 20x; however, user should determine optimal dilution factor depending on application needs.. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

### 11.2 **Saliva:**

Collect saliva using sample tube. Centrifuge samples at 800 x g for 10 minutes. Dilute samples 1:20 into 1X Diluent N or within the range of 2x – 200x; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

### 11.3 **Milk:**

Collect milk using sample tube. Centrifuge samples at 800 x g for 10 minutes. Dilute samples 1:200 into 1X Diluent N or within the range of 20x – 2000x; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

### 11.4 **Cerebrospinal fluid:**

Collect cerebrospinal fluid using sample pot. Centrifuge samples at 3,000 x g for 10 minutes. Dilute samples 1:200 into 1X Diluent N or within the range of 20x – 2000x; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -80°C for up to 3 months. Avoid repeated freeze-thaw cycles.

### 11.5 **Plasma:**

Collect plasma using one-tenth volume of 0.1 M sodium citrate as an anticoagulant. Centrifuge samples at 3000 x g for 10 minutes and collect plasma. A 20000-fold sample dilution is suggested into 1X Diluent N; however, user should

determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles (EDTA or Heparin can also be used as an anticoagulant).

### 11.6 Serum:

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3,000 x g for 10 minutes and remove serum. A 20000-fold sample dilution is suggested into 1X Diluent N; however, user should determine optimal dilution factor depending on application needs. The undiluted samples can be stored at -20°C or below for up to 3 months. Avoid repeated freeze-thaw cycles.

## 12. PLATE PREPARATION

- The 96 well plate strips included with this kit are supplied ready to use. It is not necessary to rinse the plate prior to adding reagents.
- Unused well plate strips should be returned to the plate packet and stored at 4°C.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
- Well effects have not been observed with this assay. Contents of each well can be recorded on the template sheet included in the Resources section.

## **13. ASSAY PROCEDURE**

- **Equilibrate all materials and prepared reagents to room temperature (18 - 25°C) prior to use.**
  - **It is recommended to assay all standards, controls and samples in duplicate.**
- 13.1 Prepare all reagents, working standards and samples as instructed. Equilibrate reagents to room temperature before use. The assay is performed at room temperature (18-25°C).
  - 13.2 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.
  - 13.3 Add 50  $\mu$ L of Antithrombin III Standard or sample to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for two hours. Start the timer after the last sample addition.
  - 13.4 Wash five times with 200  $\mu$ L of 1X Wash Buffer manually. Invert the plate each time and decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid. If using a machine wash six times with 300  $\mu$ L of 1X Wash Buffer and then invert the plate, decant the contents; tap it 4-5 times on absorbent paper towel to completely remove the liquid.
  - 13.5 Add 50  $\mu$ L of 1X Biotinylated Antithrombin III Detector Antibody to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate for one hour.
  - 13.6 Wash microplate as described above.
  - 13.7 Add 50  $\mu$ L of 1X SP Conjugate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Cover wells with a sealing tape and incubate

for 30 minutes. Turn on the microplate reader and set up the program in advance.

- 13.8 Wash microplate as described above.
- 13.9 Add 50  $\mu\text{L}$  of Chromogen Substrate to each well. Gently tap plate to thoroughly coat the wells. Break any bubbles that may have formed. Incubate for 20 minutes or until the optimal blue colour density develops.
- 13.10 Add 50  $\mu\text{L}$  of Stop Solution to each well. The color will change from blue to yellow. Gently tap plate to ensure thorough mixing. Break any bubbles that may have formed.
- 13.11 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.

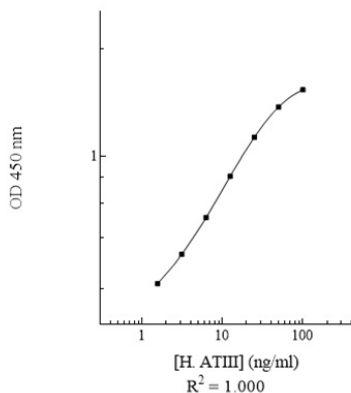
### **14. CALCULATIONS**

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. The best-fit line can be determined by regression analysis using log-log or four-parameter logistic curve-fit. Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

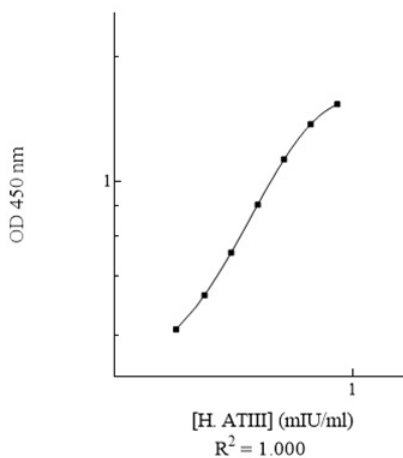
## 15. TYPICAL DATA

**TYPICAL STANDARD CURVE** – Data provided for demonstration purposes only. A new standard curve must be generated for each assay performed.

Human ATIII Standard Curve



Human ATIII Standard Curve



## 16. TYPICAL SAMPLE VALUES

### SENSITIVITY –

The minimum detectable dose of Antithrombin III is typically ~0.99 ng/mL.

### SPIKING RECOVERY –

Recovery was determined by spiking one plasma and one serum sample with different human AT3 concentrations.

Sample	Unspiked Sample (ng/ml)	Spiking Value	Expected	Observed	Recovery (%)
Plasma	9.617	12.377	21.994	19.984	91
		6.335	15.952	14.461	91
		2.894	12.511	11.517	92
Serum	15.737	12.377	28.114	25.975	92
		6.335	22.072	19.532	88
		2.894	18.631	16.848	90
<b>Average recovery</b>					91

## LINEARITY OF DILUTION –

Dilution	Average % Expected Value	
	Plasma	Serum
1:10000	94	94
1:20000	100	102
1:40000	102	103

## PRECISION –

	Intra-Assay	Inter-Assay
% CV	4.0	10.6

**17. ASSAY SPECIFICITY**

<b>Species</b>	<b>Cross Reactivity (%)</b>
Canine	3%
Equine	<1%
Monkey	2%
Mouse	None
Rat	None
Swine	3%
Rabbit	None
Bovine	None

## 18. TROUBLESHOOTING

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
Poor standard curve	Improper standard dilution	Confirm dilutions made correctly
	Standard improperly reconstituted (if applicable)	Briefly spin vial before opening; thoroughly resuspend powder (if applicable)
	Standard degraded	Store sample as recommended
	Curve doesn't fit scale	Try plotting using different scale
Low signal	Incubation time too short	Try overnight incubation at 4°C
	Target present below detection limits of assay	Decrease dilution factor; concentrate samples
	Precipitate can form in wells upon substrate addition when concentration of target is too high	Increase dilution factor of sample
	Using incompatible sample type (e.g. serum vs. cell extract)	Detection may be reduced or absent in untested sample types
	Sample prepared incorrectly	Ensure proper sample preparation/dilution
Large CV	Bubbles in wells	Ensure no bubbles present prior to reading plate
	All wells not washed equally/thoroughly	Check that all ports of plate washer are unobstructed wash wells as recommended
	Incomplete reagent mixing	Ensure all reagents/master mixes are mixed thoroughly
	Inconsistent pipetting	Use calibrated pipettes and ensure accurate pipetting
	Inconsistent sample preparation or storage	Ensure consistent sample preparation and optimal sample storage conditions (eg. minimize freeze/thaws cycles)

## RESOURCES

<b>Problem</b>	<b>Cause</b>	<b>Solution</b>
High background/ Low sensitivity	Wells are insufficiently washed	Wash wells as per protocol recommendations
	Contaminated wash buffer	Make fresh wash buffer
	Waiting too long to read plate after adding STOP solution	Read plate immediately after adding STOP solution
	Improper storage of ELISA kit	Store all reagents as recommended. Please note all reagents may not have identical storage requirements.
	Using incompatible sample type (e.g. Serum vs. cell extract)	Detection may be reduced or absent in untested sample types

### 19. NOTES

## **Technical Support**

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For all technical or commercial enquiries please go to:

<https://www.abcam.com/en-us/contact-us>

<https://www.abcam.cn/contact-us> (China)

<https://www.abcam.co.jp/contact-us> (Japan)