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Human Antithrombin III Total Antigen ELISA Kit

Catalog # HATIIIKT-TOT

Strip well format. Reagents for up to 96 tests.

Rev: June 2017

SAMPLE INSERT Refer to kit box for lot specific instructions

INTENDED USE

This human antithrombin III antigen assay is intended for the quantitative determination of total antithrombin III antigen in human plasma.

For research use only.

BACKGROUND

Antithrombin III is a glycolsylated plasma serine protease inhibitor that forms a stoichiometric complex with coagulation cascade enzymes [1]. Antithrombin III inhibits alpha-Thrombin as well as Factor Xa, IXa, XIa and XIIa with heparin enhanced kinetics [2]. Type 1 Antithrombin deficiency is characterized by decreased plasma antigen levels of antithrombin III [3].

ASSAY PRINCIPLE

Human antithrombin III will bind to the capture antibody coated on the microtiter plate. Free and complexed antithrombin III will react with the antibody on the plate. After appropriate washing steps, polyclonal anti-human antithrombin III primary antibody binds to the captured protein. Excess primary antibody is washed away and bound antibody, which is proportional to the total antithrombin III present in the samples, reacts with the secondary antibody. Following an additional washing step, TMB substrate is used for color development at 450nm. A standard calibration curve is prepared using dilutions of purified antithrombin III and is measured along with the test samples. Color development is proportional to the concentration of antithrombin III in the samples.

STANDARD CALIBRATION

Antithrombin III standard provided is calibrated against the WHO 3rd International Standard for Antithrombin, Plasma distributed by NIBSC (08/258), South Mimms, Potters Bar, Hertfordshire, UK.

Lot 1014L: 350 ng = 0.001509 IU

REAGENTS PROVIDED

- **96-well antibody coated microtiter strip plate** (removable wells 8x12) containing anti-human antithrombin III antibody, blocked and dried.
- **10X Wash Buffer:** 1 bottle of 50ml
- **Human antithrombin III standard:** 1 vial lyophilized standard
- **Anti-human antithrombin III primary antibody:** 1 vial lyophilized polyclonal antibody
- **Anti-goat horseradish peroxidase secondary antibody:** 1 vial concentrated HRP labeled antibody
- **TMB substrate solution:** 1 bottle of 10ml solution

STORAGE AND STABILITY

HRP conjugated secondary antibody must be stored at ≤ -70°C. Store all other kit components at 4°C upon arrival. Return any unused microplate strips to the plate pouch with desiccant. Reconstituted standard may be stored at -80°C for later use. Do not freeze-thaw the standard more than once. This kit should not be used beyond the expiration date

OTHER REAGENTS AND SUPPLIES REQUIRED

- Microtiter plate shaker capable of 300 rpm uniform horizontally circular movement
- Manifold dispenser/aspirator or automated microplate washer
- Microplate reader capable of measuring absorbance at 450 nm
- Pipettes and Pipette tips
- Deionized or distilled water
- Polypropylene tubes for dilution of standard
- Paper towels or laboratory wipes
- 1N H_2SO_4 or 1N HCl
- Bovine Serum Albumin Fraction V (BSA)
- Tris(hydroxymethyl)aminomethane (Tris)
- Sodium Chloride (NaCl)

PRECAUTIONS

- FOR LABORATORY RESEARCH USE ONLY. NOT FOR DIAGNOSTIC USE.
- Do not mix any reagents or components of this kit with any reagents or components of any other kit. This kit is designed to work properly as provided.
- Always pour peroxidase substrate out of the bottle into a clean test tube. Do not pipette out of the bottle as contamination could result.
- Keep plate covered except when adding reagents, washing, or reading.
- DO NOT pipette reagents by mouth and avoid contact of reagents and specimens with skin.
- DO NOT smoke, drink, or eat in areas where specimens or reagents are being handled.

PREPARATION OF REAGENTS

- **TBS buffer:** 0.1M Tris, 0.15M NaCl, pH 7.4
- **Blocking buffer (BB):** 3% BSA (w/v) in TBS
- **1X Wash buffer:** Dilute 50ml of 10X wash buffer concentrate with 450ml of deionized water.

SAMPLE COLLECTION

Collect plasma using EDTA, heparin, or citrate as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay immediately or aliquot and store at ≤ -20°C. Avoid repeated freeze-thaw cycles.

ASSAY PROCEDURE

Perform assay at room temperature. Vigorously shake plate (300rpm) at each step of the assay.

Preparation of Standard

Reconstitute standard by adding 7ml of blocking buffer directly to the vial and agitate gently to completely dissolve contents. This will result in a 50ng/ml standard solution.

Dilution table for preparation of human antithrombin III standard:

Antithrombin III concentration (ng/ml)	Dilutions
20	600µl (BB) + 400µl (std vial)
10	500µl (BB) + 500µl (20ng/ml)
5	500µl (BB) + 500µl (10ng/ml)
2	600µl (BB) + 400µl (5ng/ml)
1	500µl (BB) + 500µl (2ng/ml)
0.5	500µl (BB) + 500µl (1ng/ml)
0.2	600µl (BB) + 400µl (0.5ng/ml)
0.1	500µl (BB) + 500µl (0.2ng/ml)
0.05	500µl (BB) + 500µl (0.1ng/ml)
0.02	600µl (BB) + 400µl (0.05ng/ml)
0	500µl (BB) Zero point to determine background

NOTE: DILUTIONS FOR THE STANDARD CURVE AND ZERO STANDARD MUST BE MADE AND APPLIED TO THE PLATE IMMEDIATELY.

Standard and Unknown Addition

Remove microtiter plate from bag and add 100µl antithrombin III standards (in duplicate) and unknowns to wells. Carefully record position of standards and unknowns. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

NOTE: The assay measures antithrombin III antigen in the 0.02-20 ng/ml range. If the unknown is thought to have high antithrombin levels, dilutions may be made in blocking buffer. A 1:100,000-1:400,000 dilution for normal human plasma is suggested for best results.

Primary Antibody Addition

Reconstitute primary antibody by adding 10ml of blocking buffer directly to the vial and agitate gently to completely dissolve contents. Add 100µl to all wells. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

Secondary Antibody Addition

Briefly centrifuge vial before opening. Dilute 2µl of conjugated secondary antibody in 10ml of blocking buffer and add 100µl to all wells. Shake plate at 300rpm for 30 minutes. Wash wells three times with 300µl wash buffer. Remove excess wash by gently tapping plate on paper towel or kimwipe.

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Substrate Incubation

Add 100µl TMB substrate to all wells and shake plate for 2-15 minutes. Substrate will change from colorless to different strengths of blue. Quench reaction by adding 50µl of 1N H_2SO_4 or HCl stop solution to all wells when samples are visually in the same range as the standards. Add stop solution to wells in the same order as substrate upon which color will change from blue to yellow. Mix thoroughly by gently shaking the plate.

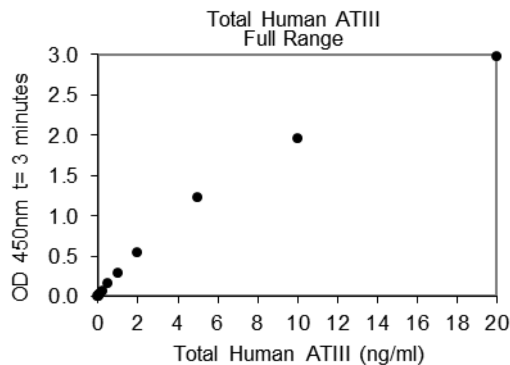
Measurement

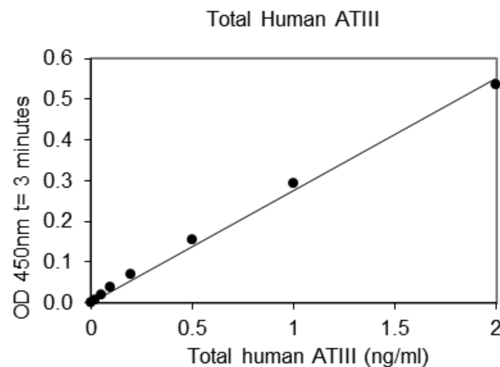
Set the absorbance at 450nm in a microtiter plate spectrophotometer. Measure the absorbance in all wells at 450nm. Subtract zero point from all standards and unknowns to determine corrected absorbance (A_{450}).

Calculation of Results

Plot A_{450} against the amount of antithrombin III in the standards. Fit a straight line through the linear points of the standard curve using a linear fit procedure if unknowns appear on the linear portion of the standard curve. Alternatively, create a standard curve by analyzing the data using a software program capable of generating a four parameter logistic (4PL) curve fit. The amount of antithrombin III in the unknowns can be determined from this curve. If samples have been diluted, the calculated concentration must be multiplied by the dilution factor.

A typical standard curve (EXAMPLE ONLY):





EXPECTED VALUES

The concentration of antithrombin III in normal human plasma by thrombin titration is 2.57 µM or 150 µg/ml [4]. An antigen concentration of 137 µg/ml in human reference plasma was determined by house testing at 1:100,000 and 1:200,000 dilutions.

PERFORMANCE CHARACTERISTICS

Sensitivity: The minimum detectable dose (MDD) was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates (range OD_{450}: 0.074-0.085) and calculating the corresponding concentration. The MDD was 0.023 ng/mL.

Intra-assay Precision: These studies are currently in progress. Please contact us for more information.

Inter-assay Precision: These studies are currently in progress. Please contact us for more information.

Recovery: These studies are currently in progress. Please contact us for more information.

Linearity: To assess the linearity of the assay, human plasma samples containing high concentrations of antigen were serially diluted to produce samples with values within the dynamic range of the assay.

Sample	1:2	1:4	1:8	1:16
n	4	4	4	4
Average % of expected	100	100	100	104
Range	91-112%	96-105%	95-106%	99-108%

Specificity: These studies are currently in progress. Please contact us for more information.

DISCLAIMER

This information is believed to be correct but does not claim to be all-inclusive and shall be used only as a guide. The supplier of this kit shall not be held liable for any damage resulting from handling of or contact with the above product.

SAMPLE INSERT
Refer to kit box for specific instructions

REFERENCES

1. Rosenberg RD and Damus PS.: J. Biol. Chem. 1973, 248: 6490-6505.
2. Olson ST, *et al*.: Methods in Enzymology. 1993, 222: 525-559.
3. Blajchman MA, *et al*. Blood, 1992, 80: 2159-2171.
4. Conard J, *et al*. Haemostasis, 1983, 13(6): 363-368.

Example of ELISA Plate Layout
96 Well Plate: 22 Standard wells, 74 Sample wells

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.02 ng/ml	0.05 ng/ml	0.1 ng/ml	0.2 ng/ml	0.5 ng/ml	1 ng/ml	2 ng/ml	5 ng/ml	10 ng/ml	20 ng/ml	
B	0	0.02 ng/ml	0.05 ng/ml	0.1 ng/ml	0.2 ng/ml	0.5 ng/ml	1 ng/ml	2 ng/ml	5 ng/ml	10 ng/ml	20 ng/ml	
C												
D												
E												
F												
G												
H												

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