

Total Human Antithrombin III Antigen Assay

Strip well format. Reagents for up to 96 tests.

For Research Use Only.

INTENDED USE

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

BACKGROUND

Antithrombin III is a glycosylated 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. After appropriate washing steps, peroxidase labeled polyclonal anti-human Antithrombin III primary antibody binds to the captured protein. Excess antibody is washed away and 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.

REAGENTS PROVIDED

- ◆ **Antibody Coated Plate:**
1-96 well immulon strip plate (8x12 removable strips) coated with anti-human Antithrombin III capture antibody, blocked, and dried
- ◆ **10X Wash Buffer:**
1 bottle of 50ml; bring to 1X using DI water
- ◆ **Human Antithrombin III standard:**
1 vial of lyophilized standard
- ◆ **Anti-human Antithrombin III primary antibody:**
1 vial of lyophilized horseradish peroxidase labeled polyclonal antibody
- ◆ **TMB substrate solution:**
1 bottle of 10ml solution

STORAGE AND STABILITY

All kit components must be stored at 4°C. Store unopened plate and any unused microtiter strips in the pouch with desiccant. Reconstituted standards and primary may be stored at -70°C for later use. **DO NOT** freeze/thaw the standards and primary antibody more than once. All other unused kit components must be stored at 4°C. Kit should be used no later than the expiration date.

REAGENTS AND EQUIPMENT REQUIRED

- 1-channel pipettes covering 0-10µl and 200-1000µl
- 12-channel pipette covering 30-300µl
- Paper towels or kimwipes
- 50ml tubes, 1.5ml centrifuge tubes
- 1N H₂SO₄

- DI water
- Magnetic stirrer and stir-bars
- Plastic containers with lids
- Microtiter plate spectrophotometer operable at 450nm
- Microtiter plate shaker with uniform horizontally circular movement up to 300rpm. (OPTIONAL)

WARNINGS

Warning – Avoid skin and eye contact when using TMB One substrate solution since it may be irritating to eyes, skin, and respiratory system. Wear safety goggles and gloves.

PRECAUTIONS

- 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.
- **DO NOT** pipette reagents by mouth.
- Always pour substrate out of the bottle into a clean test tube. **DO NOT** pipette out of the bottle as you could contaminate the substrate.
- Keep plate covered except when adding reagents, washing, or reading.
- **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:** 3% BSA in TBS buffer
- Wash buffer concentrate:** The wash buffer supplied in a 10X concentrate and must be diluted 1:10 with deionized water for use with the kit.

SPECIMEN COLLECTION

The assay measures total human Antithrombin III in the 0.01-10 ng/ml range. Samples giving human Antithrombin III levels above 10ng/ml should be diluted in blocking buffer before use. A 1:50,000 to 1:100,000 dilution for plasma is suggested for best results.

ASSAY PROCEDURE

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

Preparation of Standard:

Reconstitute standard as directed on the vial to give a 50ng/ml solution.

Dilution table for preparation of human Antithrombin III standards:

Antithrombin III concentration (ng/ml)	Dilutions
10	800µl (Blocking buffer) + 200µl (50ng/ml)
5	500µl (0ng/ml) + 500µl (10ng/ml)
2	600µl (0ng/ml) + 400µl (5ng/ml)
1	500µl (0ng/ml) + 500µl (2ng/ml)
0.5	500µl (0ng/ml) + 500µl (1ng/ml)
0.2	600µl (0ng/ml) + 400µl (0.5ng/ml)
0.1	500µl (0ng/ml) + 500µl (0.22ng/ml)
0.05	500µl (0ng/ml) + 500µl (0.1ng/ml)
0.02	600µl (0ng/ml) + 400µl (0.05ng/ml)
0.01	500µl (0ng/ml) + 500µl (0.02ng/ml)
0	500µl (Blocking buffer) 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. Add 100µl 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.

Primary Antibody Addition:

Add 10ml of blocking buffer directly to the primary antibody 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.

Substrate Incubation:

Add 100µl of TMB substrate to all wells and shake plate for 5-10 minutes. Substrate will change from colorless to different strengths of blue. Quench reaction by adding 50µl of 1N H₂SO₄ 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 and read final absorbance values at 450nm. For best results read plate immediately

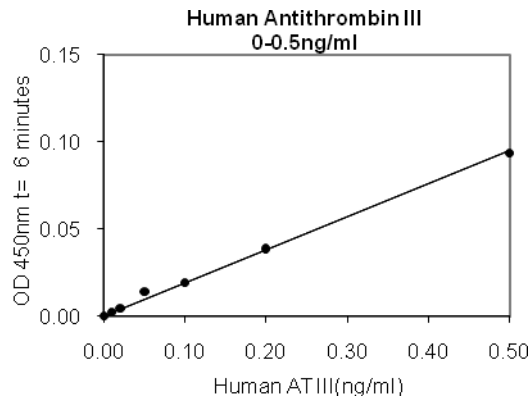
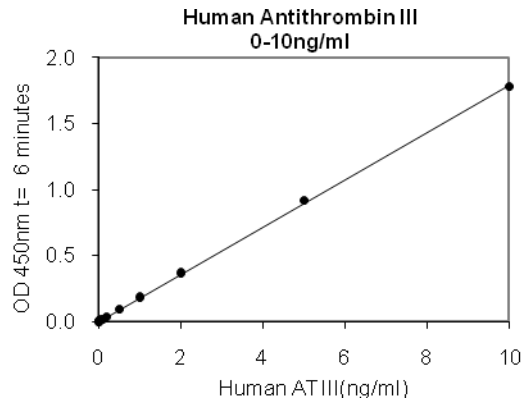
Measurement:

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 (A₄₅₀).

Assay Calibration:

Plot A₄₅₀ against the amount of human Antithrombin III in the standards. Fit a straight line through the points using a linear fit procedure. The amount of total human Antithrombin III in the unknowns can be determined from this curve.

A typical standard curve.
(EXAMPLE ONLY, DO NOT USE)



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 188 µg/ml in human reference plasma was determined by house testing at 1:100,000 dilution.

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 or from contact with the above product.

REFERENCE

1. Rosenberg RD and PS Damus. The Purification and Mechanism of Action of Human Antithrombin-Heparin Cofactor. *J. Biol. Chem.*, Sep 1973; 248:6490-6505.
2. Olson ST *et al.* Kinetic characterization of heparin-catalyzed and uncatalyzed inhibition of blood

coagulation proteinases by antithrombin. *Methods in Enzymology* 1993; 222:525-559.

3. Blajchman MA *et al.* Molecular basis of inherited human antithrombin deficiency. *Blood*, Nov 1992; 80:2159-2171.

4. Conard J *et al.* Molar antithrombin concentration in normal human plasma. *Haemostasis*, Jan 1983; 13(6):363-368.

**Example of ELISA Kit Plate Layout:
96 Well Plate**

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0.01ng/ml	0.02ng/ml	0.05ng/ml	0.1ng/ml	0.2ng/ml	0.5ng/ml	1ng/ml	2ng/ml	5ng/ml	10ng/ml	
B	0	0.01ng/ml	0.02ng/ml	0.05ng/ml	0.1ng/ml	0.2ng/ml	0.5ng/ml	1ng/ml	2ng/ml	5ng/ml	10ng/ml	
C												
D												
E												
F												
G												
H												

Standards: 22 Wells

Samples: 74 Wells