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# ab220073
# Human Antithrombin III ELISA Kit

For the quantitative competitive measurement of Human AT III in plasma, serum and milk samples.

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

## Table of Contents

# 1. Overview

The Human Antithrombin III ELISA (Enzyme-Linked Immunosorbent Assay) kit (ab220073) is designed for detection of Human Antithrombin III (AT3) in plasma, serum and milk samples. This assay employs a quantitative competitive enzyme immunoassay technique that measures AT3 in approximately 3 hours. A polyclonal antibody specific for AT3 has been pre-coated onto a 96-well microplate with removable strips. AT3 in standards and samples is competed with a biotinylated AT3 sandwiched by the immobilized antibody and streptavidin-peroxidase conjugate. All unbound material is washed away and a peroxidase enzyme substrate is added. The color development is stopped and the intensity of the color is measured.

The serine protease inhibitor antithrombin III (AT3), the most important natural inhibitor of thrombin activity, has been shown to exert marked anti-inflammatory properties. AT3 levels are positively correlated with plasma total cholesterol levels, plasma low-density lipoprotein cholesterol levels, plasma triglycerides and D-dimer levels.

## 2. Protocol Summary

Add 25 µL of Standard or Sample and
25 µL of Biotinylated Protein per well.
Incubate 2 hours.

Wash, then add 50 µL of SP Conjugate per well.
Incubate 30 minutes.

Wash, then add 50 µL of Chromogen Substrate per well.
Incubate 20 minutes.

Add 50 µL of Stop Solution per well.
Read at 450 nm immediately.

## 3. Precautions

**Please read these instructions carefully prior to beginning the assay.**

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handle with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

## 4. Storage and Stability

**Upon arrival, immediately store components of the kit at recommended temperatures up to the expiration date.**

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

## 5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

## 6. Materials Supplied

| Item | Quantity | Storage Condition |
|---|---|---|
| 100X Streptavidin-Peroxidase Conjugate | 80 µL | -20°C |
| 20X Wash Buffer Concentrate | 30 mL | +4°C |
| Anti-Human AT3 coated Microplate (12 x 8 wells) | 1 plate | +4°C |
| Antithrombin III Standard | 1 vial | +4°C |
| Biotinylated Human AT3 (Lyophilized) | 1 vial | +4°C |
| Chromogen Substrate | 7 mL | +4°C |
| 10X Diluent M Concentrate | 30 mL | +4°C |
| Sealing Tapes | 3x 1 units | +4°C |
| Stop Solution | 11 mL | +4°C |

## 7. Materials Required, Not Supplied

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

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution.
- 100 mL and 1 liter graduated cylinders.
- Absorbent paper.
- Log-log graph paper or computer and software for ELISA data analysis.

## 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 is necessary to minimize background.
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- **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. The kit contains enough reagents for 96 wells. The sample volumes below are sufficient for 48 wells (6 x 8-well strips); adjust volumes as needed for the number of strips in your experiment.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.

### 9.1 100X Streptavidin-Peroxidase Conjugate:

Spin down the SP Conjugate briefly and dilute the desired amount of the conjugate 1:100 with Diluent M. Any remaining solution should be frozen at -20°C.

### 9.2 20X Wash Buffer Concentrate:

If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Wash Buffer Concentrate 1:20 with deionized water. Store at +4°C.

### 9.3 Antithrombin III Microplate:

Ready to use. Store at +4°C.

### 9.4 Biotinylated Human AT3:

Reconstitute Biotinylated Human AT3 with 4 mL Diluent M to produce a 6-fold stock solution. Allow the biotin to sit for 10 minutes with gentle agitation prior to making dilutions. The stock solution should be further diluted 1:6 with Diluent M. Any remaining solution should be frozen at -20°C and used within 30 days.

### 9.5 Chromogen Substrate:

Ready to use. Store at +4°C.

### 9.6 10X Diluent M Concentrate :

If crystals have formed in the concentrate, mix gently until the crystals have completely dissolved. Dilute the Diluent M Concentrate 1:10 with deionized water. Store for up to 30 days at +4°C. When diluting the concentrate, make sure to rinse the bottle thoroughly to extract any precipitates left in the bottle. Mix the solution gently until the crystals have completely dissolved.

### 9.7 Sealing Tapes:

Prepare Ready to use. Store at -20°C.

**9.8 Stop Solution:**

Ready to use. Store at +4°C.

## 10. Standard Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).
- Any remaining stock solution should be stored at -20°C and used within 30 days. Avoid repeated freeze-thaw cycles.

**10.1** Reconstitution of the Human AT3 Standard vial to prepare a 4µg/mL Standard (#1)

10.1.1 First consult the AT3 Standard vial to determine the mass of protein in the vial.

10.1.2 Calculate the appropriate volume of 1X Diluent M to add when resuspending the AT3 Standard vial to produce a 4 µg/mL AT3 **Standard #1** by using the following equation:

CS = Starting mass of AT3 Standard (see vial label) (µg)
CF = 4 µg/mL AT3 Standard #1 final required concentration.
VD = Required volume of 1X Diluent M for reconstitution (µL)

Calculate total required volume 1X Diluent M for resuspension:

$$(CS/ CF) \times 1{,}000 = VD$$

Example:

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

$C_S$ = 12 µg of AT3 Standard in vial

$C_F$ = 4 µg/mL AT3 **Standard #1** final concentration

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

$$(12\ \mu g / 4\ \mu g/mL) \times 1{,}000 = 3{,}000\ \mu L$$

10.1.3 First briefly spin the AT3 Standard vial to collect the contents on the bottom of the tube.

10.1.4 Reconstitute the AT3 Standard vial by adding the appropriate calculated amount $V_D$ of 1X Diluent M to the vial to generate the 4 µg/mL AT3 **Standard #1**. Mix gently and thoroughly.

10.1.5 Allow the reconstituted 4 µg/mL AT3 **Standard #1** to sit for 10 minutes with gentle agitation prior to making subsequent dilutions.

**10.2** Label 7 tubes #2-8.

**10.3** Aliquot 300 µL of the Diluent M buffer into tubes 2-8.

**10.4** To prepare Standard #2, add 300 µL of the Standard #1 into tube #2 and mix gently.

**10.5** To prepare **Standard #3**, add 300 µL of the **Standard #2** into tube #3 and mix gently.

**10.6** Using the table below as a guide, prepare subsequent serial dilutions.

**10.7** 1X Diluent M serves as the zero standard, 0 µg/mL (tube #8).

| Tube # | Volume to dilute | Volume of diluent | Concentration (µg/mL) |
|---|---|---|---|
| 1 | 600 µL of 4 µg/mL stock solution | N/A | 4.00 |
| 2 | 300 µL of tube #1 | 300 µL | 2.00 |
| 3 | 300 µL of tube #2 | 300 µL | 1.00 |
| 4 | 300 µL of tube #3 | 300 µL | 0.50 |
| 5 | 300 µL of tube #4 | 300 µL | 0.25 |
| 6 | 300 µL of tube #5 | 300 µL | 0.125 |
| 7 | 300 µL of tube #5 | 300 µL | 0.0625 |
| 8 | N/A | 300 µL | 0 |

## 11. Sample Preparation

**11.1 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. Dilute plasma 1:300 with Diluent M and assay. 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).

**11.2 Serum**:

Samples should be collected into a serum separator tube. After clot formation, centrifuge samples at 3000 x g for 10 minutes, and remove serum. Dilute serum 1:300 with Diluent M and assay. 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. An 8-fold sample dilution is suggested into Diluent M or within the range of 2x – 80x; 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. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

**12.1** Remove excess microplate strips from the plate frame and return them immediately to the foil pouch with desiccants inside. Reseal the pouch securely to minimize exposure to water vapor and store in a vacuum desiccator.

**12.2** Add 25 µL of Antithrombin III Standard and/or sample per well and immediately add 25 µL of Biotinylated Human AT3 to each well (on top of the standard or sample) and tap plate gently to mix. Cover wells with a sealing tape and incubate for 2 hours at room temperature. Start the timer after the last addition.

**12.3** Wash five times with 200 µL of Wash Buffer manually. Invert the plate each time and decant the contents; hit 4-5 times on absorbent material to completely remove the liquid. If using a machine, wash six times with 300 µL of Wash Buffer and then invert the plate, decanting the contents; hit 4-5 times on absorbent material to completely remove the liquid.

**12.4** Add 50 µL of Streptavidin-Peroxidase Conjugate to each well and incubate for 30 minutes. Turn on the microplate reader and set up the program in advance.

**12.5** Wash the microplate as described above.

**12.6** Add 50 µL of Chromogen Substrate per well and incubate in ambient light for 20 minutes or until the optimal blue color density develops.

**12.7** Add 50 µL of Stop Solution to each well. The color will change from blue to yellow. Gently tap plate to ensure thorough mixing and break any bubbles that may have formed.

**12.8** 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.
**Δ Note:** some unstable black particles may be generated at low concentration points after stopping the reaction for about 10 minutes, which will reduce the readings

## 13. Calculations

**13.1** Calculate the mean value of the duplicate or triplicate readings for each standard, and sample.

**13.2** 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 4-parameter or log-log logistic curve-fit.

**13.3** Determine the unknown sample concentration from the Standard Curve and multiply the value by the dilution factor.

## 14. Typical Data

**Typical standard curve** – data provided **for demonstration purposes only**. A new standard curve must be generated for each assay performed.

| Standard | Human AT3 (µg/mL) | Average O.D. |
|---|---|---|
| 1 | 4.00 | 0.206 |
| 2 | 2.00 | 0.316 |
| 3 | 1.00 | 0.442 |
| 4 | 0.50 | 0.645 |
| 5 | 0.25 | 0.897 |
| 6 | 0.125 | 1.249 |
| 7 | 0.0625 | 1.526 |
| 8 | 0 | 2.134 |

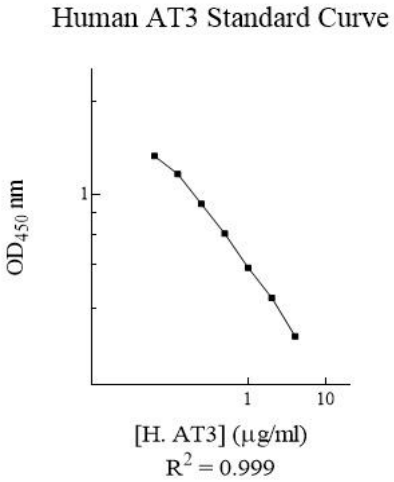


**Figure 1**.ab220073 Human Antithrombin III standard curve .

## 15. Typical Sample Values

### Reference Value

Human plasma and serum samples from healthy adults were tested (n=40). On average, AT3 level was 271 µg/mL.

| Sample | Number of measures | Average Value (µg/mL) |
|---|---|---|
| Human Pool Normal Plasma | 10 | 248 |
| Human Normal Plasma | 20 | 267 |
| Human Pool Normal Serum | 10 | 298 |

### Performance Characteristics

- The minimum detectable dose of AT3 as calculated by 2SD from the mean of a zero standard was established to be 45 ng/mL.
- Intra-assay precision was determined by testing replicates of three plasma samples in one assay.
- Inter-assay precision was determined by testing three plasma samples in twenty assays.

### Sensitivity –

The range is 0.063 µg/mL - 4 µg/mL

General sensitivity = 45 ng/mL

### Precision –

**Intra-assay precision:** (Precision within an assay) Three samples of known concentration were tested on one plate to assess intra-assay precision.

| Sample | Number of measures | CV% | Average Value (µg/ml) |
|---|---|---|---|
| 1 | 20 | 4.1 | 4.7% |
| 2 | 20 | 5.4 | |
| 3 | 20 | 4.7 | |

**Inter-assay precision:** (Precision between assays) Three samples of known concentration were tested in separate assays to assess inter-assay precision.

| Sample | Number of assays | CV% | Average Value (µg/ml) |
|---|---|---|---|
| 1 | 20 | 8.0 | 9.2 |
| 2 | 20 | 10.2 | |
| 3 | 20 | 9.4 | |

**Recovery**

| | |
|---|---|
| Standard Added Value | 0.25 – 2 µg/mL |
| Recovery % | 89 – 106% |
| Average Recovery % | 97% |

**Linearity**

– Plasma and serum samples were serially-diluted to test for linearity.

| Average Percentage of Expected Value (%) | | |
|---|---|---|
| Sample Dilution | Plasma | Serum |
| 1:150 | 106% | 104% |
| 1:300 | 98% | 99% |
| 1:600 | 94% | 95% |

**Cross-reactivity:**

| Species | Cross Reactivity (%) |
|---|---|
| Canine | None |
| Monkey | <10% |
| Equine | <1% |
| Mouse | None |
| Rat | None |
| Swine | None |
| Rabbit | None |
| Bovine | None |
| Human | 100% |

## 16. Troubleshooting

| Problem | Reason | Solution |
|---|---|---|
| **Low Precision** | Use of expired components | Check the expiration date listed before use.<br>Do not interchange components from different lots. |
| | Improper wash step | Check that the correct wash buffer is being used.<br>Check that all wells are dry after aspiration.<br>Check that the microplate washer is dispensing properly.<br>If washing by pipette, check for proper pipetting technique. |
| | Splashing of reagents while loading wells | Pipette properly in a controlled and careful manner. |
| | Inconsistent volumes loaded into wells | Pipette properly in a controlled and careful manner.<br>Check pipette calibration.<br>Check pipette for proper performance. |
| | Insufficient mixing of reagent dilutions | Thoroughly agitate the lyophilized components after reconstitution.<br>Thoroughly mix dilutions. |
| | Improperly sealed microplate | Check the microplate pouch for proper sealing.<br>Check that the microplate pouch has no punctures.<br>Check that three desiccants are inside the microplate pouch prior to sealing. |
| **Unexpectedly Low or High Signal Intensity** | Microplate was left unattended between steps | Each step of the procedure should be performed uninterrupted. |
| | Omission of step | Consult the provided procedure for complete list of steps. |
| | Steps performed in incorrect order | Consult the provided procedure for the correct order. |
| | Insufficient amount of reagents added to wells | Check pipette calibration.<br>Check pipette for proper performance. |

| | | |
|---|---|---|
| | Wash step was skipped | Consult the provided procedure for all wash steps. |
| | Improper wash buffer | Check that the correct wash buffer is being used. |
| | Improper reagent preparation | Consult reagent preparation section for the correct dilutions of all reagents. |
| | Insufficient or prolonged incubation periods | Consult the provided procedure for correct incubation time. |
| **Deficient Standard Curve Fit** | Non-optimal sample dilution | Sandwich ELISA: If samples generate OD values higher than the highest standard point (P1), dilute samples further and repeat the assay.<br>Competitive ELISA: If samples generate OD values lower than the highest standard point (P1), dilute samples further and repeat the assay.<br>User should determine the optimal dilution factor for samples. |
| | Contamination of reagents | A new tip must be used for each addition of different samples or reagents during the assay procedure. |
| | Contents of wells evaporate | Verify that the sealing film is firmly in place before placing the assay in the incubator or at room temperature. |
| | Improper pipetting | Pipette properly in a controlled and careful manner.<br>Check pipette calibration.<br>Check pipette for proper performance. |
| | Insufficient mixing of reagent dilutions | Thoroughly agitate the lyophilized components after reconstitution.<br>Thoroughly mix dilutions. |

## 17. Notes

## Technical Support



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)