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For the quantitative determination of antithrombin III in biological samples of rats.

***Please read carefully due to Critical Changes, e.g., WU]VfUrcf'VŁbWYbLfUŁcbžVŁb'i [ UŁ'ghUV']Im***

For Research Use Only. Not For Use In Diagnostic Procedures.

|                 |                                    |
|-----------------|------------------------------------|
| Catalog Number: | 41-AT3RT-E01                       |
| Size:           | 96 wells                           |
| Version:        | v3 L13.0 - ALPCO November 28, 2011 |

### **ALPCO Diagnostics**

26G Keewaydin Drive • Salem, NH 03079  
Phone: (800) 592-5726 • Fax: (603) 898-6854  
[www.alpc.com](http://www.alpc.com) • Email: [web@alpc.com](mailto:web@alpc.com)

## INTENDED USE

The antithrombin III test kit is a highly sensitive two-site enzyme linked immunoassay (ELISA) for measuring antithrombin III in biological samples of rats.

## INTRODUCTION

Antithrombin III (AT3) is a small glycoprotein produced by the liver. It is a major inhibitor of the coagulation cascade and inhibits virtually all of the coagulation enzymes, including thrombin. Deficiency of antithrombin III, either genetic or acquired can lead to thromboembolic disease.

## PRINCIPLE OF THE ASSAY

The principle of the double antibody sandwich ELISA is represented in Figure 1. In this assay, the antithrombin III present in samples reacts with the anti-antithrombin III antibodies which have been adsorbed to the surface of polystyrene microtiter wells. After the removal of unbound proteins by washing, anti-AT3 antibodies conjugated with horseradish peroxidase (HRP), are added. These enzyme-labeled antibodies form complexes with the previously bound AT3. Following another washing step, the enzyme bound to the immunosorbent is assayed by the addition of a chromogenic substrate, 3,3',5,5'-tetramethylbenzidine (TMB). The quantity of bound enzyme varies directly with the concentration of AT3 in the sample tested; thus, the absorbance, at 450 nm, is a measure of the concentration of AT3 in the test sample. The quantity of AT3 in the test sample can be interpolated from the standard curve constructed from the standards, and corrected for sample dilution.

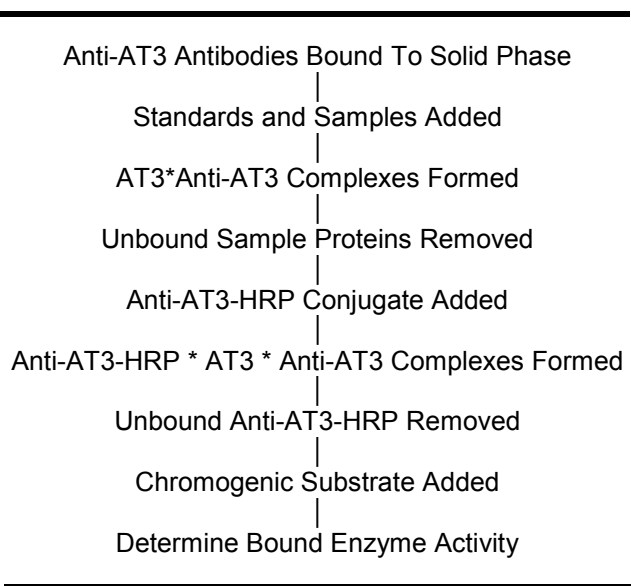


Figure 1.

## REAGENTS (Quantities sufficient for 96 determinations)

### 1. DILUENT CONCENTRATE (Assay Buffer)

One bottle containing 50 ml of a 5X concentrated diluent assay buffer.

### 2. WASH SOLUTION CONCENTRATE

One bottle containing 50 ml of a 20X concentrated wash solution.

### 3. ENZYME-ANTIBODY CONJUGATE 100X

One vial containing 150 µl of affinity purified anti-rat antithrombin III antibody conjugated with horseradish peroxidase in a stabilizing buffer.

### 4. CHROMOGEN-SUBSTRATE SOLUTION

One vial containing 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide in citric acid buffer at pH 3.3.

### 5. STOP SOLUTION

One vial containing 12 ml 0.3 M sulfuric acid. **WARNING: Avoid contact with skin.**

### 6. ANTI-RAT ANTITHROMBIN III ELISA MICROPLATE

Twelve removable eight (8) well micro well strips in well holder frame. Each well is coated with affinity purified anti-rat AT3.

### 7. RAT ANTITHROMBIN III CALIBRATOR

One vial containing a lyophilized rat antithrombin III calibrator.

## REAGENT PREPARATION

### 1. DILUENT CONCENTRATE

The Diluent Solution supplied is a 5X Concentrate and must be diluted 1:5 with distilled or deionized water (1 part buffer concentrate, 4 parts dH<sub>2</sub>O).

### 2. WASH SOLUTION CONCENTRATE

The Wash Solution supplied is a 20X Concentrate and must be diluted 1:20 with distilled or deionized water (1 part buffer concentrate, 19 parts dH<sub>2</sub>O). Crystal formation in the concentrate is not uncommon when storage temperatures are low. Warming of the concentrate to 30-35°C before dilution can dissolve crystals.

### 3. ENZYME-ANTIBODY CONJUGATE

Calculate the required amount of working conjugate solution for each microtiter plate test strip by adding 10 µl Enzyme-Antibody Conjugate to 990 µl of 1X Diluent for each test strip to be used for testing. Mix uniformly, but gently. Avoid foaming.

### 4. CHROMOGEN-SUBSTRATE SOLUTION

Ready to use as supplied.

### 5. STOP SOLUTION

Ready to use as supplied.

### 6. ANTI-RAT ANTITHROMBIN III ELISA MICROPLATE

Ready to use as supplied. Unseal Microtiter Pouch and remove plate from pouch. Remove all strips and wells that will not be used in the assay and place back in pouch and re-seal along with desiccant.

## 7. RAT ANTITHROMBIN III CALIBRATOR

Add 1.0 ml of distilled or de-ionized water to the Rat Antithrombin III Calibrator and mix gently until dissolved. The calibrator is now at a concentration of 3.164 µg/ml (**the reconstituted calibrator should be aliquoted and frozen if future use is intended**). Rat antithrombin III standards need to be prepared immediately prior to use (see chart below). Mix well between each step. Avoid foaming.

| Standard | ng/ml | Volume added to 1x Diluent | Volume of 1x Diluent |
|----------|-------|----------------------------|----------------------|
| 6        | 400   | 100 µl Rat AT3 Calibrator  | 691 µl               |
| 5        | 200   | 250 µl standard 6          | 250 µl               |
| 4        | 100   | 250 µl standard 5          | 250 µl               |
| 3        | 50    | 250 µl standard 4          | 250 µl               |
| 2        | 25    | 250 µl standard 3          | 250 µl               |
| 1        | 12.5  | 250 µl standard 2          | 250 µl               |
| 0        | 0     |                            | 500 µl               |

## STORAGE AND STABILITY

The expiration date for the package is stated on the box label.

### 1. DILUENT

The 5X Diluent Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions should be stored at 4-8°C.

### 2. WASH SOLUTION

The 20X Wash Solution Concentrate is stable until the expiration date. The 1X working solution is stable for at least one week from the date of preparation. Both solutions can be stored at room temperature (16-25°C) or at 4-8°C.

### 3. ENZYME-ANTIBODY CONJUGATE

Undiluted horseradish peroxidase anti-AT3 conjugate should be stored at 4-8°C and **diluted immediately prior to use**. The working conjugate solution is stable for up to 1 hour when stored in the dark.

### 4. CHROMOGEN-SUBSTRATE SOLUTION

The Substrate Solution should be stored at 4-8°C and is stable until the expiration date.

### 5. STOP SOLUTION

The Stop Solution should be stored at 4-8°C and is stable until the expiration date.

### 6. ANTI-RAT ANTITHROMBIN III ELISA MICROPLATE

Anti-rat AT3 coated wells are stable until the expiration date, and should be stored at 4-8°C in sealed foil pouch with desiccant pack.

## 7. RAT ANTITHROMBIN III CALIBRATOR

**The lyophilized rat antithrombin III calibrator should be stored at 4°C or frozen until reconstituted. The reconstituted calibrator should be aliquoted out and stored frozen (Avoid multiple freeze-thaw cycles).** The working standard solutions should be prepared immediately prior to use and are stable for up to 8 hours.

## INDICATIONS OF INSTABILITY

If the test is performing correctly, the results observed with the standard solutions should be within 20 % of the expected values.

## SPECIMEN COLLECTION AND HANDLING

Blood should be collected by venipuncture. The serum should be separated from the cells after clot formation by centrifugation. For plasma samples, blood should be collected into a container with an anticoagulant and then centrifuged. Care should be taken to minimize hemolysis, excessive hemolysis can impact your results. Assay immediately or aliquot and store samples at -20°C. Avoid repeated freeze-thaw cycles.

### 1. Precautions

For any sample that might contain pathogens, care must be taken to prevent contact with open wounds.

### 2. Additives and Preservatives

No additives or preservatives are necessary to maintain the integrity of the specimen. Avoid azide contamination.

### 3. Known interfering substances

Azide and thimerosal at concentrations higher than 0.1% inhibits the enzyme reaction.

## MATERIAL PROVIDED – See "REAGENTS"

## MATERIALS REQUIRED BUT NOT PROVIDED

- Precision pipette (2 µl to 1000 µl) for making and dispensing dilutions
- Test tubes
- Microtiter washer/aspirator
- Distilled or Deionized H<sub>2</sub>O
- Microtiter Plate reader
- Assorted glassware for the preparation of reagents and buffer solutions
- Timer
- Centrifuge
- Anticoagulant (for collection of plasma samples)

## ASSAY PROTOCOL

### DILUTION OF SAMPLES

The assay for quantification of antithrombin III in samples requires that each test sample be diluted before use. For a single step determination a dilution of 1:10,000 is appropriate for most serum/plasma samples. For absolute quantification, samples that yield results outside the range of the standard curve, a lesser or greater dilution might be required. **If unsure of sample level, a serial dilution with one or two representative samples before running the entire plate is highly recommended.**

1. To prepare a 1:10,000 dilution of sample, transfer 5 µl of sample to 495 µl of 1X diluent. This gives a 1:100 dilution. Next, dilute the 1:100 samples by transferring 5 µl to 495 µl of 1X diluent. You now have a 1:10,000 dilution of your sample. Mix thoroughly at each stage.

## PROCEDURE

1. **Bring all reagents to room temperature before use.**
2. Pipette 100 µl of
  - Standard 0 (0.0 ng/ml) in duplicate
  - Standard 1 (12.5 ng/ml) in duplicate
  - Standard 2 (25 ng/ml) in duplicate
  - Standard 3 (50 ng/ml) in duplicate
  - Standard 4 (100 ng/ml) in duplicate
  - Standard 5 (200 ng/ml) in duplicate
  - Standard 6 (400 ng/ml) in duplicate
3. Pipette 100 µl of sample (in duplicate) into pre-designated wells.
4. Incubate the microtiter plate at room temperature for sixty ( $60 \pm 2$ ) minutes. Keep plate covered and level during incubation.
5. Following incubation, aspirate the contents of the wells.
6. Completely fill each well with appropriately diluted Wash Solution and aspirate. Repeat three times, for a total of four washes. If washing manually: completely fill wells with wash buffer, invert the plate then pour/shake out the contents in a waste container. Follow this by sharply striking the wells on absorbent paper to remove residual buffer. Repeat 3 times for a total of four washes.
7. Pipette 100 µl of appropriately diluted Enzyme-Antibody Conjugate to each well. Incubate at room temperature for thirty ( $30 \pm 2$ ) minutes. Keep plate covered in the dark and level during incubation.
8. Wash and blot the wells as described in Steps 5 and 6.
9. Pipette 100 µl of TMB Substrate Solution into each well.
10. Incubate in the dark at room temperature for precisely ten (10) minutes.
11. After ten minutes, add 100 µl of Stop Solution to each well.
12. Determine the absorbance (450 nm) of the contents of each well. Calibrate the plate reader to manufacturer's specifications.

## STABILITY OF THE FINAL REACTION MIXTURE

The absorbance of the final reaction mixture can be measured up to 2 hours after the addition of the Stop Solution. However, good laboratory practice dictates that the measurement be made as soon as possible.

## **RESULTS**

1. Subtract the average background value from the test values for each sample.
2. Using the results observed for the standards construct a Standard Curve. The appropriate curve fit is that of a four-parameter logistics curve. A second order polynomial (quadratic) or other curve fits may also be used.
3. Interpolate test sample values from standard curve. Correct for sera dilution factor to arrive at the antithrombin III concentration in original samples.

## **LIMITATION OF THE PROCEDURE**

1. Reliable and reproducible results will be obtained when the assay procedure is carried out with a complete understanding of the information contained in the package insert instructions and with adherence to good laboratory practice.
2. Factors that might affect the performance of the assay include proper instrument function, cleanliness of glassware, quality of distilled or deionized water, and accuracy of reagent and sample pipettings, washing technique, incubation time or temperature.
3. Do not mix or substitute reagents with those from other lots or sources.