MEASUREMENT OF PROGRESSIVE ANTITHROMBIN (AT III) ACTIVITY USING ANTI IIa METHOD (#221122)

INTENDED USE:

Chromogenic assay for the quantitative determination of the "progressive" activity of Antithrombin (AT), heparin independent, in human citrated plasma using an anti IIa method, manual or automated.

ASSAY PRINCIPLE:

Antithrombin is the major physiological coagulation inhibitor. It inhibits coagulation serine esterases, especially Thrombin, Factor Xa and Factor IXa, regulates coagulation pathway and prevents from thrombosis. In the absence of heparin, Antithrombin is a "slow acting", progressive, inhibitor of coagulation serine esterases.
The antithrombin Anti-IIa method is a method based on the inhibition of a constant amount of Thrombin (IIa), by the tested antithrombin (**without addition of heparin, but with a prolonged inhibition time between Factor IIa and tested AT**), and hydrolysis of a Thrombin specific chromogenic substrate, by Thrombin in excess. pNA is then released from the substrate. The amount of pNA released is then a relation of the residual Thrombin activity. There is an inverse relationship between the concentration of AT present in the tested plasma and color development, measured at 405 nm.

As the assay is performed in the absence of heparin, it cannot be applied on plasmas from patients treated with Heparins (UFH or LMWH).

[AT + [IIa (excess)] ➔ [FIIa-AT + [residual FIIa]
[FIIa (residual)] + IIa-Subs. ➔ Peptide + pNA

REAGENTS:

BIOPHEN AT (Anti IIa) kit (#221122), used without the corresponding R3 (reagent 3: Dilution buffer with heparin, pH 8.40).

Kits are used without the R3 buffer supplied in the kits; it must be replaced by the here below buffer, without Heparin (#AR104A).

#AR104A : AT-Tris Buffer-Anti IIa (progressive Antithrombin):Special, Ready to use buffer: Tris, NaCl, EDTA Buffer, at pH 8.40, contains sodium azide (NaN3). Vials of 10 ml.

Note:

- Bovine Thrombin and BSA were prepared from bovine plasma, which was tested for the absence of infectious agents, and collected from animals free from BSE. However, no test may totally exclude the absence of infectious agents. As any product of bovine origin, it must be used with all the cautions required for handling a material potentially infectious.
- The **AT-Tris Buffer-Anti IIa (Progressive Antithrombin**) contains sodium azide, which may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water, preferentially sterile.
- Acetic Acid (20%) or Citric Acid (2%) (End point method).
- Plasma Calibrator titrated for AT (eg BIOPHEN Plasma Calibrator Ref 222101).
- Normal or Abnormal Control Plasmas titrated for AT (eg BIOPHEN Normal Control Plasma Ref 223201, and BIOPHEN Abnormal Control Plasma Ref 223301).

Material:

- Spectrophotometer, photometer or automates for chromogenic assays, with a wave-length set up at 405 nm.
- Stop Watch.
- Calibrated pipettes.

STORAGE CONDITIONS:

BIOPHEN AT (Anti IIa) kits, and **the specific buffer** without heparin, must be stored at 2-8°C, in their original packaging box. They are then stable until the expiration date printed on the box.

PREPARATION AND STABILITY OF REAGENTS:

#AR104A : AT-Tris Buffer-Anti IIa: SPECIAL Tris, NaCl, EDTA Buffer (without Heparin) Ready to use buffer. It contains Sodium Azide (0.9 g/l). This reagent is stable until the expiration date printed on the label, when stored at 2-8°C, protected from any contamination. When open: 4 weeks at 2-8°C.

221122 - R1: Bovine Thrombin, Lyophilized:
Reconstitute each vial with exactly **2.5 ml of distilled water (for test tube method)**. Let the reagent to stabilize for 30 min at Room Temperature, while shaking from time to time. Homogeneize before each use.

Stability of reconstituted Thrombin, kept in its original vial: refer to #221122 insert.

221122- R2: Chromogenic substrate specific for Thrombin (SIIa-01), lyophilized.
Reconstitute each vial with **2.5 ml of distilled water (for test tube method)**. Incubate at Room Temperature (18-25°C) for 30 min, while shaking from time to time. Homogeneize before each use.
Stability of restored substrate, kept in its original vial: refer to #221122 insert.

Cautions:

- In order to improve stability, reagents must be closed with their original screw cap following each use (white caps for Thrombin and buffer, yellow caps for SIIa-01).
- Reagents must be handled with care, in order to avoid any contamination during use.
- If the substrate becomes yellow, this indicates the presence of a contaminant. It must be rejected, and a new vial must be used.
- To incubate the reconstituted vials, for 30 minutes at room temperature, allows stabilizing the reagents, and obtaining a homogeneous reactivity over time.

Note:

- R1 and R2 vials are closed under vacuum. Remove carefully the stopper, in order to avoid any lost of powder when opening the vials.
- According to the automated method used, the reagents can be reconstituted with volumes different from those recommended. In any case, the established reactive ratios (respective reagent concentrations in the reactive milieu) between Thrombin and its substrate must be strictly respected.
- Use only reagents from kits with the same lot number. Do not mix reagents from kits with different lots when running the assay. **Reagents R1 and R2 are optimized for each lot of kits.**

PREPARATION OF PLASMA (SPECIMEN COLLECTION):

Blood (9 volumes) must be collected on 0.109M citrate anticoagulant (1 volume), with great care, in a silicon glass or a plastic tube. Sampling must be performed through a net venipuncture, avoiding any blood activation. Within 4 hours, blood must be centrifuged at 2,000-2,500 g for 15 min at room temperature (18-22°C), and plasma decanted into a plastic tube, using a plastic pipette.

Storage of plasma:

- Up to 8 hours at Room Temperature (18-25°C).
- Up to 24 hours at 2-8°C.
- Up to 1 month frozen at –20°C or below (before use, place the sample at 37°C to obtain complete thawing).

TEST PROCEDURE:

This progressive Antithrombin assay is designed for being used in chromogenic methods, automated (***provided that the instrument is able to manage the 1 hour incubation step used***), but it can also be used for end point manual methods. The assay is performed at the controlled temperature of 37°C and the color development is measured at 405 nm. As the assay is a progressive measurement of AT, performed without heparin, the inhibition step of thrombin by AT is prolonged **and extended to 1 hour**.

CALIBRATION:

This chromogenic progressive AT assay can be calibrated with **BIOPHEN Plasma Calibrator (#222101),** which has a well defined Antithrombin concentration, "**C**". The following calibration range must be prepared as follows:

% AT	%AT	Plasma Calibrator (µl)	AT-Tris Buffer-Anti IIa (Progressive Antithrombin) (µl)
0	0	0	500
C:8	12.5	500 of C3	500
C:4	25	500 of C2	500
C:2	50	500 of C	500
C	100	1000	0

Note:C:16 dilution can be introduced if required.

ASSAY PROTOCOL:

Manual Method:

Dilute the tested samples, the controls and the calibration solutions **1:20 (test tube)** with AT-Tris Buffer-Anti IIa special Buffer (#AR104A)

In a microplate well, or in a **plastic** tube preincubated at **37°C**, introduce:

Reagents	Microplate	Test Tube
Calibrators, Controls or tested plasmas:	100 µL **Plasma 1 :20**	400 µL **Plasma 1 :20**
R1 : Thrombin preincubated **at 37°C**	50 µL	200 µL
Mix and Incubate for **exactly 1 hour at 37°C**, then introduce:		
R2: (SIIa-01), Substrate preincubated **at 37°C**	50 µL	200 µL
Mix and Incubate for **1 min at 37°C, exactly**		
Stop the reaction by introducing:		
Citric Acid (20g/L) or 20 % Acetic Acid	100 µL	400 µL
Mix and measure the optical density at **405nm** against the sample blank.		

The yellow color obtained is stable for 2 hours.

The sample blank is obtained by mixing the reagents in the opposite order from that of the test i.e.: Acid , (SIIa-01) substrate, diluted plasma, Thrombin.

Measure the Absorbance at 405 nm (A405). Subtract the sample blank from the A405 obtained for the assay.

Note:

- If higher or lower reactive volumes are required for the method used, the same respective proportions for each reagent concentration, and for tested plasmas, must be strictly respected, in order to keep the assay performances.
- Do a sample blank in presence of highly lipemic, icteric or hemolysed plasmas, or if the plasmas has a "color" different from the usual one**.**

QUALITY CONTROL:

The use of quality control plasmas allows validating the calibration curve, as well as the homogeneous reactivity of the progressive AT chromogenic assay from run to run and from series to series, when using a same lot of reagents. Various control plasmas are available:

BIOPHEN Normal Control Plasma: (ref 223201).
BIOPHEN Abnormal Control Plasma: (ref 223301).

LIMITATIONS OF THE PROCEDURE:

- For a better accuracy, samples measured ≤20% can be tested at the 1:10 dilution, and obtained results divided by 2; for samples measured >120%, the 1:40 dilution can be used and obtained results multiplied by 2. If a different dilution factor from the standard 1:20 is used, the concentration must be corrected by the complementary dilution factor, i.e. x2 for 1:40, or x0.5 for 1:10).
- In two-point kinetics methods, there is no significant interference for haemoglobin concentrations <5 mg/ml, bilirubin concentrations <0.6 mg/ml, and triglycerides <1.25 mg/ml, when added to normal plasma. These analytes can interfere in absorbance readings: in these cases, individual plasma blanks are necessary when end-point manual methods are used (acid stopped).
- **As the assay is performed in the absence of heparin, it cannot be applied on plasmas from patients treated with Heparins (UFH or LMWH).**
- Thrombin inhibitors (eg Hirudin, Argatroban) present in the tested sample may lead to overestimation of AT concentration. In order to get the optimal assay performances, the working instructions must be carefully observed. Each laboratory should verify performances in its exact working conditions.

RESULTS:

For the end point method, using a linear graph paper plot, on abscissae, the Antithrombin concentration (%) and on ordinates the corresponding absorbance (A405).

The Antithrombin concentration in the tested sample is directly obtained on the calibration curve (indirect relationship)

- Results are expressed as % AT.
- Using automated methods, the Antithrombin concentrations are directly calculated by the analyzer, respectively to the calibration curve.
- Dynamic range: 0.0 to 1.0 IU/ml, ie 0 to 100%
- AT Detection threshold: 0.10 IU/ml, ie ≤10% AT
- Standardization: International or Internal reference for Antithrombin.