HYPHEN BioMed
155 rue d'Eragny, 95000 Neuville-sur-Oise, France
Tél : +33 (0)1 34 40 65 10
Fax : +33 (0)1 34 48 72 36
www.hyphen-biomed.com
info@hyphen-biomed.com

CE
IVD

BIOPHEN™ AT (Anti-IIa)

Ref 221122

(R1, R2 : 2 x 2.5 mL, R3 : 2 x 25 mL)

Chromogenic method for quantitative determination of antithrombin (AT) activity in human plasma.

English, last revision: 09-2024

INTENDED USE:

The BIOPHEN™ AT (Anti-IIa) kit is a chromogenic method for *in vitro* quantitative determination of antithrombin (AT) activity, in presence of an excess of heparin, on human citrated plasma or purified milieu using an anti-IIa method, manual or automated.

SUMMARY AND EXPLANATION:

The AT congenital deficiencies induce spontaneous thromboembolic deficiencies. These congenital deficiencies in AT are classed in 4 different groups[1]:

- Type I: Decreased AT concentration and decreased AT activity; the most frequent case.
- Type II RS (Reactive Site): Normal AT concentration and decreased biological activity; a protein abnormality is present at the active site.
- Type II HBS (Heparin Binding Site): Normal AT concentration, normal AT activity in the absence of heparin, but decreased in its presence and an abnormality of AT-Heparin link.
- Type II PE (Pleiotropic Effects): Decreased AT concentration and decreased biological activity; non functional protein and at a lowered level.

Help in the diagnosis of congenital deficiencies or acquired AT deficiency.
Assay of AT (anti-IIa activity) in plasma, or purified milieu when required.

PRINCIPLE:

Antithrombin (AT) 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. When complexed to heparin, AT becomes a potent and fast acting inhibitor of coagulation serine esterases. This anti-IIa assay thus measures the heparin-dependent anti-IIa activity of AT[2,3,4,5].
The BIOPHEN™ AT (Anti-IIa) method is an assay based on the inhibition of a constant and in excess amount of Thrombin (IIa), by the tested AT in presence of an excess of heparin, and hydrolysis of a Thrombin specific chromogenic substrate, by Thrombin in excess. pNA is then released from the substrate. The amount of pNA released (measured by absorbance at 405 nm) is then a relation of the residual Thrombin quantity. There is an inverse relationship to the concentration of AT present in the tested sample.

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

REAGENTS:

R1: Bovine Thrombin, lyophilised in presence of stabilizers. Contains BSA.
2 vials of 2.5 mL.
R2: Chromogenic substrate specific for Thrombin (SIIa-01), lyophilized.
2 vials of 2.5 mL.
R3: Specific dilution buffer with heparin, at pH 8.40, liquid form.
2 vials of 25 mL.

WARNINGS AND PRECAUTIONS:

- Biological products must be handled with all necessary precautions and considered as being potentially infectious.
- A yellow color indicates a contaminated substrate. Discard the vial and use a new one.
- Waste should be disposed of in accordance with applicable local regulations.
- Use only the reagents from the same batch of kits. Do not mix reagents from different kit batches when performing an assay; they are optimized for each batch of kits.
- Handle the reagents with care to avoid contamination during use. If possible, avoid reagent evaporation during use by limiting the liquid-air exchange surface. Evaporation reduces the reagent's stability in the analyzer.
- To preserve reagent stability, seal the vials after use with their respective caps.
- Aging studies, conducted over a 3-week period at 30°C, show that the reagents can be shipped at room temperature over a short period of time, without degradation.
- The bovine plasma used to prepare the BSA and bovine thrombin has been tested by recorded methods and is certified free of infectious agents, in particular the causative agent of bovine spongiform encephalitis.
- Create a plasma blank if this latter is icteric, lipaemic, haemolysed, or if its color differs from the standard plasmas.
- When employing the kinetic method, use ΔOD 405 instead of OD 405.
- The bovine thrombin concentration is adjusted for each lot for optimal reactivity.
- For *in vitro* diagnostic use.

REAGENT PREPARATION AND STABILITY:

The reagents are lyophilized under a vacuum in their vials. To avoid any product loss when opening the vial of lyophilized reagents, gently remove the freeze-drying stopper.

R1: Reagent 1: **Bovine Thrombin**
Reconstitute the contents of each vial with exactly **2.5 mL distilled water**, shake vigorously until fully dissolved. Allow to stabilize for 30 min. at room temperature (18-25°C), shaking occasionally.
Homogenize the reagent prior to use.
Reagent stability after reconstitution, excluding any contamination or evaporation, and stored in the original vial, is of:

- **15 days** at 2-8°C.
- **7 days** at room temperature (18-25°C).
- **6 months** frozen at -20°C or less*

R2: Reagent 2: **Chromogenic substrate specific for Thrombin**
Reconstitute the contents of each vial with exactly **2.5 mL distilled water**, shake vigorously until fully dissolved. Allow to stabilize for 30 min. at room temperature (18-25°C), shaking occasionally.
Homogenize the reagent prior to use.
Reagent stability after reconstitution, excluding any contamination or evaporation, and stored in the original vial, is of:

- **15 days** at 2-8°C.
- **7 days** at room temperature (18-25°C).
- **6 months** frozen at -20°C or less*

*Thaw only once, as rapidly as possible at 37°C, adapting the incubation period to the volume of reagent**.** The stability of the thawed reagent should be checked under laboratory work conditions.

R3: Reagent 3: **Specific dilution buffer with heparin**
Ready to use. Allow to stabilize for 30 minutes at room temperature (18-25°C), before use.
Homogenize the reagent prior to use.
Reagent stability after opening, excluding any contamination or evaporation, and stored in the original vial, is of:

- **At least 7 days** at 2-8°C.
- **7 days** at room temperature (18-25°C).
- **Do not freeze.**

STORAGE CONDITIONS:

Unopened reagents should be stored at 2-8°C in their original packaging. Under these conditions, they can be used until the expiry date printed on the kit.

REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water.
- 20% acetic acid or 2% citric acid (end point method).
- Specific calibrator or reference material for Antithrombin (international or internal, or normal citrated reference human plasma pool) and controls with known titration, such as:

Product Name	Reference
BIOPHEN™ Plasma Calibrator	222101
BIOPHEN™ Normal Control Plasma	223201
BIOPHEN™ Abnormal Control Plasma	223301

Materials:

- Spectrophotometer or automatic instrument for chromogenic assays.
- Stopwatch; Calibrated pipettes.

SPECIMEN COLLECTION AND PREPARATION:

Specimens should be prepared and stored in accordance with applicable local guidelines (for the United States, see the CLSI H21-A5[6] guidelines for further information concerning specimen collection, handling and storage).

- Specimens:

Human plasma obtained from anticoagulated blood (trisodium citrate).

- Collection:

The blood (9 volumes) should be carefully collected onto the trisodium citrate anticoagulant (1 volume) (0.109 M) by clean venipuncture. Discard the first tube.

- Centrifugation:

Within 2 hours, use a laboratory-validated method to obtain platelet-poor plasma, for example at least 15 minutes at 2500 g at room temperature (18-25°C) and allow the plasma to settle in a plastic tube.

- Plasma storage[7, 8]:
  - 4 hours at room temperature (18-25°C).
  - 1 month at -20°C.
  - 24 months at -70°C.

Frozen plasma specimens should be thawed rapidly at 37°C, then shaken thoroughly and tested immediately. Resuspend any precipitate by shaking vigorously immediately after thawing and before use.

PROCEDURE:

The kit can be used for kinetic, automated or manual (endpoint) methods. Perform the test at **37°C** and read color intensity at **405nm**.

Automated methods:
Applications for the various analyzers are available on request. **See the specific application and specific precautions for each analyzer.**

Assay method:
1. Reconstitute the calibrators and controls as indicated in the specific instructions.
When calibration is performed with a commercially available plasma calibrator (eg BIOPHEN™ Plasma Calibrator) or with internal or international reference material for AT, the **1:40** dilution corresponds to the indicated concentration (C%) of AT. The 100% concentration (in the assay conditions) is then obtained by using the following dilution factor: **40 x C :100**.
The kit can also be calibrated with a normal pooled citrated plasma (at least 30 normal individuals, aged between 18 and 55 years, and free of any medication or disease), with the assigned value of 100% AT. The assay includes a standard plasma dilution of **1:40** in R3 buffer, which by definition represents the 100% of AT.

Prepare 2 mL of the **1:40** dilution of the normal plasma pool, or of a dilution (**40 x C :100**) of the titrated AT calibrator, in the specific dilution buffer (R3). This corresponds to 100% AT (noted C1); the calibration curve can then be obtained by preparing serial dilutions as follows:

Calibrator	C1	C2	C3	C4	C5
AT (%)	100	50	25	12.5	0
Volume calibrator	1000µL of C1	500µL of C1	500µL of C2	500µL of C3	0µL
Volume Buffer R3	0 µL	500µL	500µL	500µL	500µL

In order to get the full assay performances, the calibration curve must be prepared just before running the assay.

The calibration curve can also be performed using a reference AT material (international standard or internal standard preparation). Predilute the preparation in appropriate buffer to exactly 1 IU/mL, then dilute it **1:40** with R3 for obtaining the 100% AT concentration (noted C1) and prepare the calibration range as describe above.

2. Dilute the specimens and controls in R3 buffer, as described in the table below:

Specimens	Reference	Dilution
Controls	223201 / 223301	**1:40**
Specimen	n.a.	**1:40**

For AT in purified milieu, the tested specimen must be pre-diluted in appropriate buffer, to obtain an expected AT concentration in the range 0.2-1.0 IU/mL. Then dilute it **1:40** with R3 for the assay. The AT concentration is then expected in the range 20-100% (the measured concentration must then be multiplied by the "pre-dilution" factor).

Establish the calibration curve and test it with the quality controls. If stored at room temperature (18-25°C), test the diluted specimens within 2 hours. The exact calibrator and control concentrations for each batch are indicated on the flyer provided with the kit.

3. Dispense the following to the wells of a microplate, or to a plastic tube incubated at **37°C**:

	Microplate	Volume
Calibrators, Controls or **diluted** tested specimen (1:40)	100 µL	400 µL
R1: Thrombin Pre-incubated at **37°C**	50 µL	200 µL
Mix and incubate **at 37°C for exactly 1 minute**, then add the following:		
R2: Thrombin Substrate Pre-incubated at **37°C**	50 µL	200 µL
Mix and incubate at 37°C for **1 minute exactly**		
Stop the reaction by adding:		
Citric acid (2%)*	100 µL	400 µL
Mix and measure the optical density at **405nm** against the corresponding blank.		

*Or acetic acid (20%). The yellow color is stable for 2 hours.
The specimen blank is obtained by mixing the reagents in the reverse order to that of the test: Citric acid (2%), R2, R1, diluted specimen**.**
Measure the optical density at **405 nm**. Subtract the measured blank value from the absorbance measured for the corresponding test.

Kinetics mode:
The assay can be realized by a kinetics method by measuring the change in absorbance in a shorter time following the addition of the substrate (ΔOD405). In this case, no need to subtract the sample blank, or to stop the reaction.

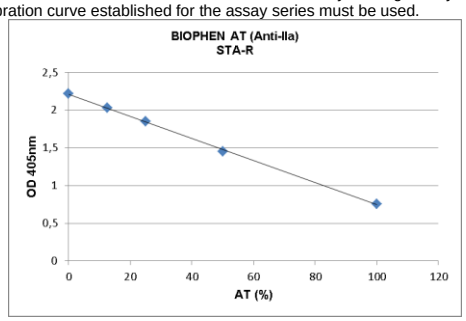
If a reaction volume other than that specified above is required for the method used, the ratio of volumes must be strictly observed to guarantee assay performance. The user is responsible for validating any changes and their impact on all results.

CALIBRATION:

The plasma calibrator covering the dynamic test range is available from HYPHEN BioMed (see the "REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED" paragraph) and can be used to establish the calibration curve.

- The calibration range is from about 0 to 100% AT (ie about 0 to 1 IU/mL).

The calibration curve shown below, obtained on STA-R® analyzer, is given by way of example only. The calibration curve established for the assay series must be used.



QUALITY CONTROL:

The use of quality controls serves to validate method compliance, along with between-test assay homogeneity for a given batch of reagents.
Include the quality controls with each series, as per good laboratory practice, in order to validate the test. A new calibration curve should be defined, preferably for each test series, and at least for each new reagent batch, or after analyzer maintenance, or when the measured quality control values fall outside the acceptable range for the method.
Each laboratory must define its acceptable ranges and verify the expected performance in its analytical system.

RESULTS:

- For the manual endpoint method, plot the calibration curve Lin-Lin, with the OD 405 nm along the Y-axis and the Antithrombin concentration, expressed as %, along the X-axis.
- The concentration of Antithrombin in the test specimen is directly inferred from the calibration curve, if the standard dilution is used. The measured concentration must be multiplied by the complementary dilution factor, if any.
- Results are expressed in %.
- The results should be interpreted according to the patient's clinical and biological condition

LIMITATIONS:

- To ensure optimum test performance and to meet the specifications, the technical instructions validated by HYPHEN BioMed should be followed carefully. The laboratory is responsible for validating any changes made to these instructions for use.
- Any reagent presenting an unusual appearance or showing signs of contamination must be rejected.
- Any suspicious samples or those showing signs of activation must be rejected.
- Any plasma displaying a coagulum or showing signs of contamination must be rejected.
- For a better accuracy, samples measured ≤20% can be tested at the **1:20** dilution, and obtained results divided by 2; for samples measured >120%, the **1:80** dilution can be used and obtained results multiplied by 2. If a different dilution factor from the standard **1:40** is used, the concentration must be corrected by the complementary dilution factor, i.e. x 2 for **1:80**, or x 0.5 for **1:20**).
- For the possible influence of interferences, refer to specific application for the analyzer used (no significant effect is observed in two-point kinetics methods (STA-R®) for bilirubin concentration up to 60 mg/dL, hemoglobin concentration up to 500 mg/dL and triglycerides concentration up to 125 mg/dL, by plasma overload tests).
- Thrombin inhibitors (eg: Hirudin, Argatroban, Dabigatran…) present in the tested sample may lead to overestimation of AT concentration.
- The assay can also be performed on purified milieu, using corresponding appropriate calibration.
- AT measured in some variants can present variation when tested with the various AT activity assays. Depending on the variant (and treatment), discrepancy between AT activity anti IIa and anti Xa assays is reported as well as very rare normal result.

EXPECTED VALUES:

The normal plasma Antithrombin level in the adult population is usually in the range of about 80 to 120%. However, each laboratory has to determine its own normal range.
AT concentration ≤ 70% indicates the presence of a deficiency, which must be confirmed by another test and/or by testing another plasma sample from the patient. AT concentration is decreased during pregnancy, oral contraceptive therapy, DIC and in case of liver disease. It is also normally low in neonates.

PERFORMANCE:

- The detection threshold is evaluated on the calibration curve by measuring the "apparent" AT concentration, which corresponds to the mean A405 value obtained for a sample free of AT (buffer alone) minus 3 Standard Deviations (SD). This detection threshold is < 0.10 IU/mL, ie <10% AT.
- On STA-R®, the measuring range is from about 20 to 120% of AT.
- **Specificity:** An AT depleted specimen is measured <5% AT. No interference of heparin (UFH, LMWH) at usual therapeutic doses: the assay can be performed on samples from patients under heparin therapy. Bovine thrombin is used, so that Heparin Cofactor II interference can be neglected at usual levels. With immediate action of AT due to the presence of Heparin and short reaction time, progressive AT activity (eg α2-macroglobulin) does not impact the assay. Plasmin, if present in the sample, is blocked by aprotinin present in R1.
- Performance studies were conducted internally on 1 batch of reagent for N=10 using a STA-R®. Performance was assessed using laboratory controls for a control level. The following results were obtained:

Control	Intra assay				Inter assays			
	n	Mean	CV%	SD	n	Mean	CV%	SD
Normal Control	10	92	1.9	1.79	10	91	5.7	5.14
Abnormal Control	10	53	1.7	0.88	10	54	3.4	1.81

REFERENCES:

1. Cooper P.C. *et al.* The phenotypic and genetic assessment of antithrombin deficiency. Int J Lab Hematol. 2011.
2. Leslie B. *et al.* Investigation of the anticoagulant mechanism of a covalent antithrombin-heparin complex. The Journal of Biological Chemistry. 1999.
3. Tsiang M *et al.* Functional requirements for inhibition of Thrombin by Antithrombin III in the presence and absence of heparin. The journal of Biological Chemistry.1997.
4. Tollefsen D.M. Laboratory Diagnosis of Antithrombin and Heparin Cofactor II deficiency. Seminars in Thromb haemost. 1990.
5. Mortensen J.Z. Inherited ATIII deficiency. Fast and slow inactivation of thrombin and Factor Xa Thromb. Res. 1984.
6. CLSI Document H21-A5: "Collection, transport, and processing of blood specimens for testing plasma -based coagulation assays and molecular hemostasis assays; approved guideline". 2008.
7. Woodhams B. *et al.* Stability of coagulation proteins in frozen plasma. Blood coagulation and Fibrinolysis. 2001.
8. Mauge L. and Alhenc-Gelas M. Stabilité pré-analytique des paramètres de la coagulation: revue des données disponibles. Ann Biol Clin. 2014.
9. Van Cott E.M *et al.* Recommendations for clinical laboratory testing for antithrombin deficiency; Communication from the SSC of the ISTH, J Thromb Haemost 2020.

SYMBOLS:

Symbols used and signs listed in the ISO 15223-1 standard, see Symbol definitions document.

Changes compared to the previous version.