HYPHEN BioMed
www.hyphen-biomed.com
155, rue d'Eragny
95000 NEUVILLE SUR OISE
FRANCE
Tel. : +33 (0)1 34 40 65 10
Fax : +33 (0)1 34 48 72 36
info@hyphen-biomed.com

BIOPHEN™ Antithrombin

CE IVD

REF 221102 R1 R2 2 x 2.5 mL; R3 2 x 5 mL

REF 221105 R1 R2 4 x 5 mL; R3 4 x 10 mL

Chromogenic assay for measuring Antithrombin in plasma with an Anti-Xa method.

English, last revision: 03-2019

INTENDED USE:

The BIOPHEN™ Antithrombin kits are chromogenic assays for the *in vitro* quantitative determination of the heparin cofactor activity of Antithrombin (AT) in human citrated plasma using an anti Xa method, manual or automated.

SUMMARY AND EXPLANATION:

Technical:

AT is the major physiological coagulation inhibitor. It inhibits coagulation serine esterases, especially Thrombin, Factors Xa (FXa) and IXa, AT regulates coagulation pathway and prevents from thrombosis. When complexed to heparin, AT becomes a potent and fast acting inhibitor of coagulation serine esterases[1].

Clinical:

Congenital or acquired AT deficiencies is correlated with an increased risk of thrombosis.

Spontaneous thromboembolic diseases are observed in presence of congenital deficiencies. These congenital deficiencies are classed in 4 different groups[2,3,4]:

- **Type I**: Decreased AT concentration and decreased AT activity; this is 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.
- **Type II** (Pleiotropic): Decreased AT concentration and activity; non functional protein and at a lowered level.

PRINCIPLE:

BIOPHEN™ Antithrombin assay is a kinetic method based on the inhibition of FXa, which is at a constant concentration and in excess, by AT in presence of heparin. The remaining FXa is then measured by its amydolitic activity on a FXa specific chromogenic substrate, which releases pNA. The amount of pNA generated is inversely proportional to the AT concentration present in the tested plasma[5].

Due to the assay's insensitivity to heparin, plasmas from patients on heparin therapy may be tested.

Heparin + AT ➔[AT Hep.]
[AT Hep.] + [Excess FXa] ➔ [FXa-AT-Hep.] + [Remaining FXa]
[Remaining FXa] + SXa-11 ➔ Peptide + pNA

REAGENTS:

R1 **Bovine Factor Xa**, lyophilized. Contains BSA.

R2 **Factor Xa specific chromogenic substrate (SXa-11),** lyophilized.

R3 **Tris-Heparin Buffer,** at pH 7.85, ready to use. Contains small amounts of sodium azide (0.9 g/L).

BIOPHEN™ Antithrombin 2,5

REF **221102** ➔ R1 **2 vials of 2.5 mL** (contains about 5 µg of Factor Xa).
R2 **2 vials of 2.5 mL** (contains about 3.75 mg of SXa-11).
R3 **2 vials of 5 mL.**

BIOPHEN™ Antithrombin 5

REF **221105** ➔ R1 **4 vials of 5 mL** (contains about 10 µg of Factor Xa).
R2 **4 vials of 5 mL** (contains about 7.5 mg of SXa-11).
R3 **4 vials of 10 mL.**

WARNINGS AND PRECAUTIONS:

- Some reagents provided in these kits contain materials of animal origin. Users of reagents of these types must exercise extreme care in full compliance with safety precautions in the manipulation of these biological materials as if they were infectious.
- Bovine Factor Xa was 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.
- In contact with lead or copper pipes, sodium azide can generate explosive compounds.
- Waste should be disposed of in accordance with applicable local regulations.
- Use only the reagents from the same batch of kits.
- Aging studies conducted over a 3-week period at 30°C, show that the reagents can be shipped at room temperature without degradation.
- This device of *in vitro* diagnostic use is intended for professional use in the laboratory.

REAGENT PREPARATION:

Gently remove the freeze-drying stopper, to avoid any product loss when opening the vial.

Reconstitute the contents of each vial with exactly:

REF **221102** ➔ R1 **2.5 mL of** R3
R2 **2.5 mL of distilled water**

REF **221105** ➔ R1 **5 mL of** R3
R2 **5 mL of distilled water**

Shake vigorously until complete dissolution while avoiding formation of foam and load it directly on the analyzer following application guide instruction.

For manual method, allow to stabilize for 30 minutes at room temperature (18-25°C), homogenize before use.

R3 Reagent is ready to use.

STORAGE AND STABILITY:

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.

R1 R2 Reagent stability after reconstitution, free from any contamination or evaporation, and stored closed, is of:

- **3 months** at 2-8°C.
- **7 days** at room temperature (18-25°C).
- **Do not freeze.**
- **Stability on board of the analyzer: see the specific application.**

A yellow color indicates a contaminated substrate. Discard the vial and use a new one.

R3 Reagent stability after opening, free from any contamination or evaporation, and stored closed, is stable until the expiration date printed on the label, when stored at 2-8°C.

REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water.
- 20% acetic acid or 2% citric acid (end point method).
- Physiological Saline (0.9% NaCl).
- AT-Tris buffer-Anti Xa for variant method.
- Specific Plasma Calibrators and controls with a known concentration such as:

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

Also refer to the specific application guide of the analyzer used.

Materials:

- Spectrophotometer or automatic analyzer for chromogenic assays.
- Stopwatch; Calibrated pipettes; silicon glass or plastic test tubes or microplate.

SPECIMEN COLLECTION AND PREPARATION:

The blood (9 volumes) should be carefully collected onto the trisodium citrate anticoagulant (1 volume) (0.109 M, 3.2%) by clean venipuncture. Discard the first tube.

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

For plasma storage, please refer to references[6,7].

PROCEDURE:

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

Assay method:

1. Reconstitute the calibrator and controls as indicated in the specific instructions. For the calibration curve, dilute the calibrators in physiological saline buffer as described below in order to establish the calibration range ("C" defines the concentration of AT):

Calibrator (222101)	C	C:2	C:4	0
Calibrator volume	500µL	250µL	125µL	0µL
Physiological saline volume	0µL	250µL	375µL	500µL

2. Dilute the specimens in physiological saline buffer, as described in the table below:

Specimens	Reference	Dilution
Control	223201	**1:20**
Control	223301	**1:20**
Calibrator	222101	**1:20**
Specimen	N.A.	**1:20**

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
Specimen, control or calibrator diluted	40 µL	80 µL
R1 **Factor Xa** Preincubated at **37°C**	100 µL	200 µL
Mix and incubate at 37°C, for **1 minute**, then add the following:		
R2 **SXa-11 Substrate** Preincubated at **37°C**	100 µL	200 µL
Mix and incubate at 37°C for **exactly:**	1 min	1 min
Stop the reaction by adding:		
Citric acid (2%)*	100 µL	200 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 from that of the test i.e.: 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.

Create a plasma blank if sample is icteric, lipaemic, haemolysed, or if its color differs from the standard plasmas.

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.

For an automated method, application guides are available on request. See specific application guide and specific precautions for each analyzer.

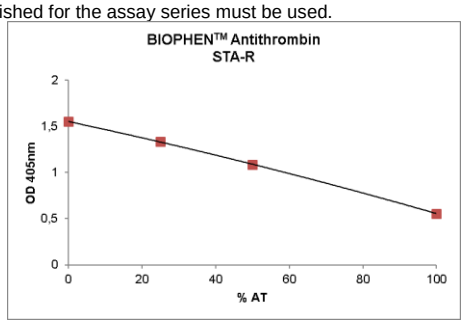
Variant method:
For the identification of type II abnormality, HBS (Heparin Binding Site), a variant method can be used. The Bovine Factor Xa vial must be restored with 2.5 mL of Tris-buffer, without heparin (AT-Tris buffer-Anti Xa). A calibration curve must be done with the Plasma Calibrator and the patient AT activity (HBS) is directly read on the curve. The specific protocol is available upon request (D750-30/AT-prog/Anti Xa).
In presence of the HBS variant, the patient has a normal AT activity with this method.

CALIBRATION:

The BIOPHEN™ Antithrombin assay can be calibrated for the assay of heparin cofactor activity of AT. The calibrator covering the calibration 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 about 5 to 120%.

The calibration curve shown below 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 established, 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 acceptance range for the method.
Each laboratory must define its acceptance ranges and verify the expected performance in its analytical system.

RESULTS:

- For the manual endpoint method, plot the calibration curve, with the OD 405 nm along the Y-axis and the AT concentration, expressed as %, along the X-axis. When employing the kinetic method, use ΔOD 405 instead of OD 405.
- The concentration of Antithrombin (%AT) in the test specimen is directly inferred from the calibration curve, when the standard dilution is used.
- If other dilutions are used, the level obtained should be multiplied by the additional dilution factor used.
- 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.
- 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.
- As the assay is an Anti-Xa method, there is no interference of Heparin Cofactor II, ∝2-macroglobulin or α1-Antitrypsin[1].

EXPECTED VALUES:

By definition, the 100% AT concentration corresponds to the concentration in a normal human citrated plasma pool, obtained by pooling plasmas from healthy males or females aged from 18 to 55 years, and out of any medication. The plasma level of AT of healthy adult is generally between 80 and 120%. 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.
The AT concentration is decreased during pregnancy and during oral contraceptive therapy.

PERFORMANCES:

- The detection threshold is calculated by measuring the "apparent" A405 obtained for an AT deficient sample less 3 standard deviations (SD). This detection threshold is ≤ 5%.
- The assay working range is 5 to 120%.
- Performance study was performed in-house using 1 lot reagent on ACL 7000. Inter assay (12 runs per sample) and intra assay performances were evaluated using samples with variable AT concentrations. Following data were obtained:

Control	Intra assay				Inter assays			
	n	Mean	CV%	SD	n	Mean	CV%	SD
1	10	107.7	0.7	0.8	12	109.5	2.6	2.8
2	10	69.4	0.7	0.5	12	69.6	2.5	1.7
3	10	51.0	0.9	0.5	12	50.3	3.7	1.9

- Interferences:
  In two-point kinetic methods, there is no interference for haemoglobin concentrations up to 5 mg/ml, for bilirubin concentrations up to 0.1 mg/mL, and for plasma from hyperlipaemic patients. These analytes can interfere in absorbance readings: in these cases, individual plasma blanks are necessary when end-point manual methods are used (acid stopped).
  There is no known drug interference in the assay.
  Also refer to the specific application guide of the analyzer used.

REFERENCES:

1. Mann K.G. Biochemistry and Physiology of blood coagulation. Thrombosis and Haemostasis. 1999.
2. Patnaik M.M. and Moll S. Inherited antithrombin deficiency: a review. Haemophilia. 2008.
3. Amiral J and Seghatchian J. Revisiting antithrombin in health and disease, congenital deficiencies and genetic variants, and laboratory studies on α and β forms. Transfus Apher Sci. 2018.
4. Khor B. and Van Cott E.M. Laboratory tests for antithrombin deficiency. American Journal of Hematology. 2010.
5. Odegard O.R. *et al*. Heparin cofactor activity measured with an amidolytic method. Thromb. 1975.
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. Mauge L. and Alhenc-Gelas M. Stabilité pré-analytique des paramètres de la coagulation: revue des données disponibles. Ann Biol Clin. 2014.

SYMBOLS:

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

R1 H315 : Causes skin irritation.
H319 : Causes serious eye irritation.
H335 : May cause respiratory irritation.

Changes compared to the previous version.