

BIOPHEN AT (LRT) Ref A221111-RUO

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

**FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**



Manufactured By: HYPHEN BioMed

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INTENDED USE:

BIOPHEN AT (LRT) kit is a chromogenic assay for the quantitative determination of the heparin cofactor activity of Antithrombin (AT) in human citrated plasma^{1,2,3} using an anti Xa method⁴, manual or automated. Reagents of BIOPHEN AT (LRT) kit are liquid, ready to use (LRT = Liquid reagent Test). **This kit is for research use only and should not be used for patient diagnosis or treatment.**

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^{5,6}. When complexed to heparin, Antithrombin becomes a potent and fast acting inhibitor of coagulation serine esterases.

BIOPHEN AT (LRT) assay is a kinetic method based on the inhibition of Factor Xa, which is at a constant concentration and in excess, by Antithrombin in presence of heparin. The remaining Factor Xa is then measured by its amygdolitic activity on a Factor Xa specific chromogenic substrate, which releases pNA. The amount of pNA generated is inversely proportional to the Antithrombin concentration present in the tested plasma.

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: Reagent 1: Bovine Factor Xa

Bovine Factor Xa, liquid form.

4 vials containing about 11ml of Factor Xa reagent, at pH 7.85, containing heparin and sodium azide.

R2: Reagent 2: SXa-11

Chromogenic substrate, specific for Factor Xa (SXa-11), liquid form.

4 vials containing about 4 ml of SXa-11.

Note:

- 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. As any product of bovine origin, this factor Xa must be used with all the cautions required for handling a material potentially infectious.
- Bovine factor Xa (R1) 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).
- Physiological saline (0.9% NaCl).
- Plasma Calibrator (BIOPHEN Plasma Calibrator Ref A222101).
- Normal or Abnormal Control Plasmas (BIOPHEN Normal Control Plasma Ref A223201, and BIOPHEN Abnormal Control Plasma Ref A223301).

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 (LRT) kits 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:

R1 : Reagent 1: Bovine Factor Xa

Ready to use.

Let the reagent to stabilise for 30 min at Room Temperature, before use.

Shake gently before use.

Stability of Factor Xa reagent, open, kept in its original vial, and provided that any bacterial contamination is avoided during use:

- 3 months at 2-8°C.
- 7 days at Room Temperature.
- Do not freeze.

R2 : Reagent 2: Factor Xa specific Chromogenic substrate (SXa-11)

Ready to use.

Incubate at Room Temperature (18-25°C) for 30 minutes, before use.

Shake gently before use.

Stability of the substrate, open, kept in its original vial, and provided that any bacterial contamination is avoided during use:

- 3 months at 2-8°C.
- 7 days at Room Temperature.
- Do not freeze.

Cautions:

- In order to improve stability, reagents must be closed with their original screw cap following each use.
- 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 vials, for 30 minutes at room temperature, allows stabilising the reagents, and obtaining a homogeneous reactivity over time.

Note:

- According to the automated method used, the volumes can be different from those recommended for the manual method. In any case, the established reactive ratios (respective reagent concentrations in the reactive milieu) between Factor Xa 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 0.109 M 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 3,000 g for 20 min at 18°C or below, 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, thaw for 15 min. in a water bath at 37°C).

Refer to NCCLS document H21-A2 for further instructions on specimen collection, handling and storage.

TEST PROCEDURE:

BIOPHEN AT (LRT) kit is designed for being used in kinetic methods, automated, but it can also be used for end point manual methods. Adaptations for the various automates are available upon request. The assay is performed at the controlled temperature of 37°C and the colour development is measured at 405 nm.

CALIBRATION:

BIOPHEN AT (LRT) kit can be calibrated with the **BIOPHEN Plasma Calibrator (ref A222101)**, which has a well defined Antithrombin concentration, "C". The following calibration range must be prepared as follows:

% AT	Plasma Calibrator (µl)	Physiological Saline (µl)
0	0	500
C/4	125	375
C/2	250	250
C	500	0



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ASSAY PROTOCOL:

Manual Method:

Microplate: Dilute the tested samples, the controls and the calibration solutions **1:20** with physiological saline (0.15 M Sodium Chloride).

Test tube: Dilute the tested samples, the controls and the calibration solutions **1:10** with physiological saline (0.15 M Sodium Chloride).

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

Reagents	Microplate	Test Tube
Diluted Calibrators, Controls or tested plasmas	20 µL	30 µL
R1 : Facteur Xa preincubated at 37°C	100 µL	300 µL
Mix and Incubate at 37°C, for 90 seconds , then introduce:		
R2: Sxa-11 Substrate preincubated at 37°C	35 µL	100 µL
Mix and Incubate for at 37°C, for 120 seconds exactly		
Stop the reaction by introducing:		
Citric Acid (20g/L)	100 µL	500 µL
Mix and measure the optical density at 405nm against the sample blank.		

The yellow colour 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.: Citric Acid (20 g/L), Sxa-11 substrate, diluted plasma, Factor Xa.

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

Automated methods:

Detailed instrument settings for a variety of automated instruments are available upon request.

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 "colour" 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 BIOPHEN AT (LRT) 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 A223201).

BIOPHEN Abnormal Control Plasma: (ref A223301).

LIMITATIONS OF THE PROCEDURE:

- As the assay is an Anti-Xa method, there is no interference of Heparin Cofactor II, α 2-macroglobulin or α 1-Antitrypsin^{7,8,9}.
- No significant interference was noticed using the manual method for haemoglobin concentrations up to 2.5 mg/ml, and for bilirubin concentrations up to 0.1 mg/ml in plasma. Some analytes can interfere in absorbance readings: in these cases, individual plasma blanks are necessary when end-point manual methods are used (acid stopped).
- In order to get the optimal performances of the assay, the procedural instructions must be strictly respected.
- The results obtained should be for research purposes only and not used for patient diagnosis or treatment.**

RESULTS:

There is an inverse relationship between the absorbance measured at 405 nm (**A405**) and the ATIII concentration in the assayed plasma. The assay is linear on the range 0 to 120% ($r^2 \geq 0.98$).

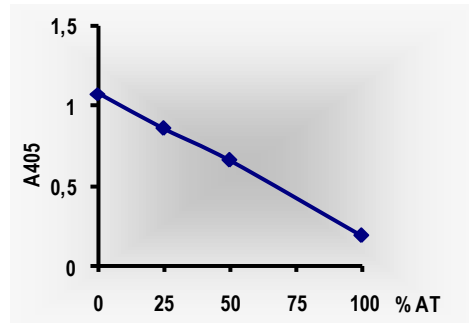
For the end point method, using a linear graph paper plot, on abscissae, the Antithrombin concentration (%) and on ordinates the corresponding absorbance (**A405**).

The Antithrombin concentration in the tested sample is directly obtained on the calibration curve. Results are expressed as %.

- Using automated methods, the Antithrombin concentrations are directly calculated by the analyser, respectively to the calibration curve.
- The dynamic range is from 10 to 120 %.

EXAMPLE OF CALIBRATION CURVE:

The calibration curve below is indicated as an example only. Only the calibration curve generated for the series of measures performed must be used.



VALIDATION OF CALIBRATION CURVE:

The calibration curve is acceptable when the concentrations measured for the Control Plasmas are within the acceptance range.

PERFORMANCES AND CHARACTERISTICS:

- The detection threshold is calculated by measuring the "apparent" A405 obtained for an Antithrombin deficient sample less 3 standard deviations (SD). This detection threshold is $\leq 10\%$.
- Example of reproducibility performances obtained for samples with variable Antithrombin concentrations, using the end point manual method:

	Intra Assay CV%	N	Inter Assay CV%	N
Sample 1 (90% AT)	0.99	10	2.73	10
Sample 2 (56% AT)	1.38	10	0.57	10

REFERENCES:

- 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* vol. 272, N°18 12024-12029 (1997)
- Odegard O R et al. Heparin cofactor activity measured with an amidolytic method. *Thromb res* 6, 287-294 (1975).
- Mann K.G. Biochemistry and Physiology of blood coagulation. *Thrombosis and Haemostasis* vol 82 N° 2 165-174 (1999).
- Demers C et al. An Antithrombin III assay based on factor Xa inhibition provides a more reliable test to identify congenital antithrombin III deficiency than an assay based on thrombin inhibition. *Thromb Haemostas* 69, 231-235 (1993).
- Leslie B. et al. Investigation of the anticoagulant mechanism of a covalent antithrombin-heparin complex. *The Journal of Biological Chemistry* vol. 273 N° 52 34730-34736 (1999).
- Mortensen J.Z. Inherited ATIII deficiency. Fast and slow inactivation of thrombin and Factor Xa. *Thromb. Res.*, 33, 511-515 (1984).
- Tran T H et al. Influence of heparin cofactor II (HCII) in the determination of Antithrombin III (AT). *Thromb Res* 40, 571-576 (1985).
- Tollefsen D.M. Laboratory Diagnosis of Antithrombin and Heparin Cofactor II deficiency. *Seminars in Thromb haemost* 16, 162-168 (1990).
- Andersson N-E et al. New chromogenic ATIII activity kit which is insensitive to heparin cofactor II and designed for use on automated instruments. *Thromb Haemost* 65, 912 (1991).