



BIOPHEN Antithrombin 5

Ref 221105

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

For *in vitro* diagnostic use only

HYPHEN BioMed

ZAC Neuville Université – 155, rue d'Eragny
95000 Neuville-sur-Oise – France
Tél. : 01 34 40 85 10 – Fax : 01 34 48 72 36
www.hyphen-biomed.com



Last revision: 12/07/2010

INTENDED USE:

BIOPHEN Antithrombin 5 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.

CLINICAL APPLICATIONS:

Diagnosis of congenital or acquired Antithrombin deficiencies.

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 Antithrombin 5 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 amyloidolytic 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, lyophilised:

4 vials containing about 10 µg of Factor Xa (to be reconstituted with 5 ml of Tris-Heparin buffer (R3)).

R2: Reagent 2: SXa-11

Chromogenic substrate, specific for Factor Xa (SXa-11), lyophilised:

4 vials containing 7.5 mg of SXa-11 (to be reconstituted with 5 ml of distilled water).

R3: Reagent 3: Tris-Heparin Buffer

Ready to use, Tris-Heparin Buffer, at pH 7.85, contains sodium azide (NaN₃).

4 vials of 10 ml.

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.
- Tris-heparin Buffer (R3) 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 222101).
- Normal or Abnormal Control Plasmas (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 Antithrombin 5 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

Reconstitute each vial with exactly 5 ml of Tris-Heparin Buffer (R3). Let the reagent to stabilise for 30 min at Room Temperature, before use.

Shake gently before use.

Stability of reconstituted Factor Xa, kept in its original vial:

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

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

Reconstitute each vial with 5 ml of distilled water. Incubate at Room Temperature (18-25°C) for 30 min.

Shake gently before use

Stability of restored substrate, kept in its original vial:

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

R3: Reagent 3: Tris-Heparin Buffer

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.

Cautions:

- In order to improve stability, reagents must be closed with their original screw cap following each use (white caps for factor Xa and buffer, yellow caps for SXa-11).
- 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 stabilising 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 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 Antithrombin 5 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 Antithrombin 5 kit can be calibrated with the **BIOPHEN Plasma Calibrator (ref 222101)**, 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

ASSAY PROTOCOL:

Manual Method:

Dilute the tested samples, the controls and the calibration solutions 1:20 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
Calibrators, Controls or tested plasmas, diluted 1:20	40 µL	80 µL
R1 : Facteur Xa preincubated at 37°C	100 µL	200 µL
Mix and Incubate for 1 min at 37°C, then introduce:		
R2: SXa-11 Substrate preincubated at 37°C	100 µL	200 µL
Mix and Incubate for 1 min at 37°C, exactly		
Stop the reaction by introducing:		
Citric Acid (20g/L)	100 µL	200 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:

Adaptations to the various analysers (STA-R, BCT, BCS, etc...) are available upon request. Reconstitution volumes can vary according to the automate used. Refer to each specific adaptation and specific cautions for each instrument.

Note:

- If higher or lower reactive volumes than those indicated here above are required for the method used, the same respective proportions between reagent concentrations and volumes used, must be adhered to, in order to maintain the assay performances.
- Run a sample blank in presence of highly lipemic, icteric or haemolysed plasmas, or if the plasmas has a "colour" different from the usual one.

QUALITY CONTROL:

To use of quality control plasmas allows validating the calibration curve, as well as the homogeneous reactivity of the BIOPHEN Antithrombin 5 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:

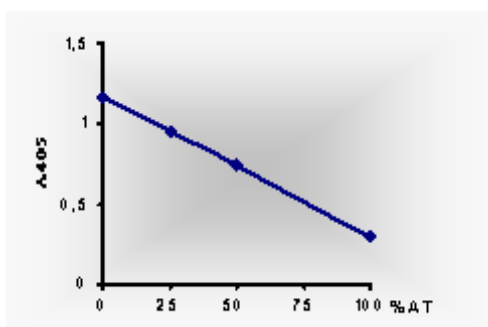
- There is no known drug interference in the assay.
- As the assay is an Anti-Xa method, there is no interference of Heparin Cofactor II, α -2-macroglobulin or α -1-Antitrypsin^{7,8,9}.
- In two-point kinetic methods, there is no interference for haemoglobin concentrations up to 5 mg/ml, for bilirubin 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).
- In order to get the optimal performances of the assay, the procedural instructions must be strictly respected.

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. Results are expressed as % of a normal plasma pool.
- Using automated methods, the Antithrombin concentrations are directly calculated by the analyser, respectively to the calibration curve.
- The dynamic range is from 5 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.

Note:

- A new calibration curve must be carried out for each new batch of reagents, after an important maintenance of the instrument, or if measured values are not in compliance with the one expected.
- Each laboratory can define its own acceptance range, according to the protocols and instruments used.

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 $\leq 5\%$.
- Example of Intra-Assay and Inter-Assay reproducibilities obtained for samples with variable Antithrombin concentrations (ACL):

Samples	AT concentrations %	Intra-Assay CV%	N	Inter-Assay CV%	N
Sample 1	109	0.73	10	2.57	12
Sample 2	69	0.66	10	2.49	12
Sample 3	51	0.92	10	3.72	12

EXPECTED VALUES:

By definition, the 100 % Antithrombin 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 relationship between released pNA measured as absorbance at 405nm, and the level of AT is linear in the 80-120% range of normal plasma.

Antithrombin concentration $\leq 70\%$ indicates the presence of a deficiency, which must be confirmed by another test and/or by testing another plasma sample from the patient.

The Antithrombin concentration is decreased during pregnancy and during oral contraceptive therapy.

CLINICAL INFORMATION:

Spontaneous thromboembolic diseases are observed in presence of congenital deficiencies. These congenital deficiencies are classed in 4 different groups:

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

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 5 mL of Tris-buffer, without heparin [Reference AR103A : AT-Tris-buffer (Anti Xa)]. A calibration curve must be done with the Plasma Calibrator and the patient Antithrombin activity (HBS) is directly read on the curve. The specific protocol is available upon request (D.750.30/AT-prog/Anti Xa).

In presence of the HBS variant, the patient has a normal Antithrombin activity with this method.

REFERENCES:

1. 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)
2. Odegard O R et al. Heparin cofactor activity measured with an amidolytic method. *Thromb res* 6, 287-294 (1975).
3. Mann K.G. Biochemistry and Physiology of blood coagulation. *Thrombosis and Haemostasis* vol 82 N° 2 165-174 (1999).
4. 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).
5. 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).
6. Mortensen J.Z. Inherited ATIII deficiency. Fast and slow inactivation of thrombin and Factor Xa. *Thromb. Res.*, 33, 511-515 (1984).
7. Tran T H et al. Influence of heparin cofactor II (HCII) in the determination of Antithrombin III (AT). *Thromb Res* 40, 571-576 (1985).
8. Tollefsen D.M. Laboratory Diagnosis of Antithrombin and Heparin Cofactor II deficiency. *Seminars in Thromb haemost* 16, 162-168 (1990).
9. 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).