# BIOPHEN Antithrombin 5

REF 221105

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

IVD

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

HYPHEN BioMed

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## INTENDED USE:

The 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 method[4], manual or automated.

## SUMMARY AND EXPLANATION:

Antithrombin is the major physiological coagulation inhibitor. It inhibits coagulation serine esterases, especially Thrombin, Factors Xa and IXa, Antithrombin 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.
The clinical application is the diagnosis of congenital or acquired Antithrombin deficiencies.
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.

## PRINCIPLE:

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 amydolitic 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, lyophilized.
**4 vials of 5 mL** (about 10µg /vial).

R2 **Reagent 2:** Factor Xa specific chromogenic substrate (SXa-11), lyophilized.
**4 vials of 5 mL** (about 7.5mg/vial).

R3 **Reagent 3:** Tris-Heparin Buffer, at pH 7.85, ready to use.
**4 vials of 10 mL**.

Reagent 3 contains small amounts of sodium azide (0.9 g/L), see WARNINGS AND PRECAUTIONS.

## WARNINGS AND PRECAUTIONS:

- Biological products must be handled with all necessary precautions and considered as being potentially 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.
- 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.
- Create a plasma blank if a plasma is icteric, lipaemic, haemolysed, or if its color differs from the standard plasmas.
- For *in vitro* diagnostic use.

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

## 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 Factor Xa.**
Reconstitute the contents of each vial with exactly **5 mL of Tris-Heparin Buffer (R3)**, 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:

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

R2 **Reagent 2**: **Factor Xa specific chromogenic substrate (SXa-11).**
Reconstitute the contents of each vial with exactly **5 mL of 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:

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

R3 **Reagent 3: Tris-Heparin Buffer.**
Ready to use.
Stability of reagent, provided that any contamination or evaporation is avoided, kept in its original vial is stable until the expiration date printed on the label, when stored at 2-8°C:

## 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).
- Physiological Saline (0.9% NaCl).
- AT-Tris buffer-Anti Xa (AR103) 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 |

***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 guidelines for further information concerning specimen collection, handling and storage[11]).

- 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) with great care 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:
  - 4 hours at room temperature (18-25°C).
  - 1 month at -20°C.
  - 18 months at -70°C[10].

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 (end point) methods. Perform the test at **37°C** and read color intensity at **405 nm**.

**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 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 Antithrombin):

| 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 validate 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, calibrator or control 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 sample 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.

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.

**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 (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 (D750-30/AT-prog/Anti Xa).
In presence of the HBS variant, the patient has a normal Antithrombin activity with this method.

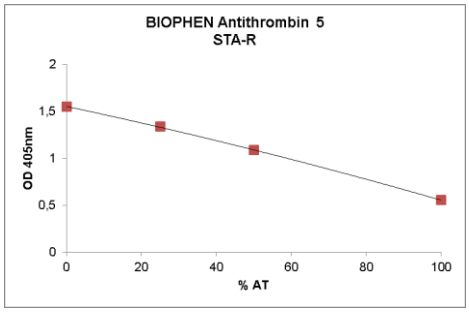
It is responsibility of the user to validate any modifications and their impact on all assay results.

## CALIBRATION:

The BIOPHEN Antithrombin 5 assay can be calibrated for the assay of heparin cofactor activity of Antithrombin (AT). A calibrator that covers the test dynamic range is available at HYPHEN BioMed (see table in the REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED section) and can be used to establish the calibration curves.
Using a linear scale:

- The dynamic range is from 5 to 120%.

The calibration curve shown below, obtained on STA-R instrument with the calibrator BIOPHEN Plasma Calibrator (222101) 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 end point method, plot the calibration curve, with the OD 405 nm along the Y-axis and the Antithrombin concentration expressed as %, along the X-axis.
- Using automated methods, the Antithrombin concentrations are directly calculated by the analyzer, respectively to the calibration curve.
- The Antithrombin concentration in the tested specimen is directly deduced from the calibration curve.
- Results are expressed in % AT.
- 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.
- 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, ∝2-macroglobulin or α1-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).

## 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 ≤ 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.

## PERFORMANCES:

- 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 ≤ 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:

| Samples | Intra assay | | | | Inter assay | | | |
|---|---|---|---|---|---|---|---|---|
| | n | Mean (%AT) | CV% | SD | n | Mean (%AT) | CV% | SD |
| 1 | 10 | 107 | 0.7 | 0.8 | 12 | 109 | 2.6 | 2.8 |
| 2 | 10 | 69 | 0.7 | 0.5 | 12 | 69 | 2.5 | 1.7 |
| 3 | 10 | 51 | 0.9 | 0.5 | 12 | 50 | 3.7 | 1.9 |

## REFERENCES: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11. CLSI Document H21-A5: "Collection, transport, and processing of blood specimens for testing plasma -based coagulation assays and molecular hemostasis assays; approved guideline". Fifth Edition, 28, 5, 2008


## SYMBOLS:

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