

BIOPHEN AT (ANTI-IIa)

A221122-RUO

Quantitative determination of antithrombin (AT) activity, in human plasma, using an anti-IIa chromogenic assay

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



Manufactured By: HYPHEN BioMed

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INTENDED USE:

BIOPHEN AT anti-IIa kit is an in vitro chromogenic assay for the quantitative determination of antithrombin (AT) activity, in presence of an excess of heparin, in human citrated plasma or purified milieu, using an anti-IIa method, manual or automated. **This assay is for research use only and should not be used for patient diagnosis or treatment.**

SPECIMEN:

Plasma prepared from citrated anticoagulated blood.
Possibly purified milieu, where AT anti-IIa activity must be measured.

ASSAY 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 antithrombin (anti-IIa) activity of AT.

The antithrombin Anti-IIa method is a method based on the inhibition of a constant 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 is then a relation of the residual Thrombin activity. There is an inverse relationship between the concentration of AT and color development, measured at 405 nm.

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

REAGENTS:

R1: Reagent 1: Bovine Thrombin, lyophilized in presence of stabilizers.
2 vials (each vial to be reconstituted with 2.5 mL of distilled water).

R2: Reagent 2: Chromogenic substrate specific for Thrombin (SIIa-01), lyophilized.
2 vials (each vial to be reconstituted with 2.5 mL of distilled water).

R3: Reagent 3: Specific dilution buffer with heparin, at pH 8.40, containing sodium azide.
2 vials of 25 mL. Ready to use.

Warning:

- Bovine Serum Albumin (BSA) and bovine thrombin were prepared from bovine plasma, which was tested for the absence of infectious agents, and collected from animals free from BSE. However, no assay may warrant the total absence of infectious agents. Any product of biological origin must then be handled with all the required cautions, as being potentially infectious.
- **Thrombin concentration is adjusted for each lot for providing the right reactivity in the assay.**
- Sodium azide (0.9 g/l) (R3 buffer) 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).
- Plasma Calibrator with a known antithrombin activity (eg **BIOPHEN Plasma Calibrator #A222101**), Or Reference material for Antithrombin (international (eg NIBSC) or internal)
Or Normal citrated reference human plasma pool
- Normal and Abnormal Quality Control Plasmas, with a known antithrombin activity (eg **BIOPHEN Normal Control Plasma #A223201**, and **BIOPHEN Abnormal Control Plasma #A223301**).

Material:

Spectrophotometer, photometer or automates for chromogenic assays, with a wave-length set up at 405 nm. Stop watch. Calibrated pipettes.

STORAGE CONDITIONS:

Reagents must be stored at 2-8°C, in their original packaging box. They are then stable until the expiration date printed on the box.

Note: Stability studies for 3 weeks at 30°C show that the reagents can be shipped at room temperature for a short period without damage.

PREPARATION AND STABILITY OF REAGENTS:

Note: Refer to each specific instrument adaptation.

R1: Reagent 1: Bovine Thrombin

Reconstitute each vial with **exactly 2.5 mL** of distilled water. Shake thoroughly until complete dissolution of the content (vortex). Let to homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time. Homogenize the content before each use.

Stability of restored reagent R1, provided that any contamination or evaporation is avoided, kept in its original vial or in a plastic tube:

- 15 days at 2-8°C.
- 7 days at room temperature (18-25 °C).
- 6 months at -20°C or below (before use, place in a water bath at 37°C for at least 15 min, to obtain complete thawing).

R2: Reagent 2: Substrate

Reconstitute each vial with **exactly 2.5 mL** of distilled water. Shake thoroughly until complete dissolution of the content (vortex). Incubate at room temperature (18-25°C) for 30 minutes, while shaking the vial from time to time (vortex). Check that the substrate is totally dissolved before use. Homogenize the content before each use.

Stability of restored R2, provided that any contamination or evaporation is avoided, kept in its original vial or in a plastic tube:

- 15 days at 2-8°C.
- 7 days at room temperature (18-25 °C).
- 6 months at -20°C or below (before use, place in a water bath at 37°C for at least 15 min, to obtain complete thawing).

R3: Reagent 3: Dilution buffer with heparin.

Ready to use buffer. Shake before each use. Stability of the buffer, protected from any contamination or evaporation:

- In its original vial, until the expiration date printed on the label, at 2-8°C.
- When open, at least 7 days at 2-8 °C or RT.

Cautions:

- In order to improve stability, reagents must be closed with their original stopper and screw cap following each use (white cap for R1, yellow cap for R2, and white cap for buffer R3).
- 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.
- Incubating the reconstituted vials at RT allows stabilizing the reagents, and obtaining a homogeneous reactivity.
- Take care to limit as much as possible any evaporation of the reagents during use, eg. by using chimneys.

Note:

- R1 and R2 vials are closed under vacuum. Remove carefully the stopper, in order to avoid any loss 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 thrombin and its substrate must be adhered to.
- **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, R2 are optimized for each lot of kits.**

TESTED SPECIMEN:

Blood (9 volumes) must be collected on 0.109M 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 2,000-2,500 g for 15 min at room temperature (18-22°C), 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, place the sample at 37°C to obtain complete thawing).

Refer to GEHT or NCCLS/CLSI recommendations for further instructions on specimen collection, handling and storage. Discard or take specific caution on any plasma presenting an unusual aspect (haemolysed, lipaemic...).

TEST PROCEDURE:

This kit is designed as a two stage method, for being used with automated methods, but it can also be used for end point manual methods. Adaptations to 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:

- Calibration is performed with a normal pooled citrated plasma (made with plasmas from at least 30 normal individuals, males or females, 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. By definition, this latter dilution of the pool represents the 100 % AT activity. The dynamic range is from 0 to 100 % AT.

- Or the kit can also be calibrated with a commercially available plasma calibrator, titrated for AT concentration, or with internal or international reference material for AT. If calibration is performed with a commercially available plasma calibrator, with a known AT concentration (C%): the 1:40 dilution corresponds to the indicated "C" concentration. In this case, the 100% concentration is obtained (in the assay conditions) by using the following dilution factor: 40 x C :100.

Prepare 2 ml of the 1:40 dilution of the normal plasma pool, or of a (40 x C :100) dilution of the AT reference standard, 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:

Standard	C1	C2	C3	C4	C5
% AT	100	50	25	12.5	0
Vol of AT standard	1000µL of C1	500µL of C1	500µL of C2	500µL of C3	0µL
Vol of 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 (with the known AT content) in appropriate

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buffer to exactly 1 unit/ml, then dilute it 1:40 with R3 for obtaining the 100% AT concentration and prepare the calibration range as for a plasma pool titrating 100% AT.

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.

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

TESTED SAMPLES AND CONTROLS:

Samples and controls are assayed at the 1: 40 dilution in R3 Buffer.

ASSAY PROTOCOL:

Into the microwell or in a **plastic** test tube, incubated at 37°C, introduce:

Reagents	Microplate	Test Tube
Calibrators, or diluted tested specimen (1:40 dilution) or Controls	100 µl	400 µl
R1 : Thrombin preincubated at 37°C	50 µl	200µl
Mix and incubate for exactly 1 minute at 37°C, then introduce:		
R2: Thrombin Substrate preincubated at 37°C	50 µl	200 µl
Mix and incubate for exactly 1 min at 37°C,		
Stop the reaction by introducing:		
Citric Acid (20g/L), or 20 % Acetic Acid	100 µl	400 µl
Mix and measure the Absorbance at 405nm against the corresponding blank.		

The yellow colour obtained 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 (20 g/L), thrombin substrate, thrombin, diluted specimen.

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

• Kinetics mode:

The assay can be read using a kinetics mode. In this case the change in absorbance is recorded for a shorter time following the addition of substrate. There is then no need to subtract the sample blank, or to stop the reaction. The results are obtained using the change in absorbance (ΔA_{405}) for calibrators and tested specimen.

• Automated methods:

Adaptations to the various analysers are available upon request. The assay is then performed kinetically. The reaction does not require to be stopped and sample blanks are automatically subtracted. 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.
- Take appropriate cautions and 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:

Using commercially available quality control plasmas, titrated for AT, allows validating the calibration curve, as well as the homogeneous reactivity from run to run, when using a same lot of reagents. The calibration curve is acceptable when the concentrations measured for controls are within the acceptance range. Various control plasmas are available: **BIOPHEN Normal Control Plasma (#A223201)** and **BIOPHEN Abnormal Control Plasma (#A223301)**. Each laboratory should verify its own target value and acceptance range, in the exact working conditions, for each new lot of controls.

Note:

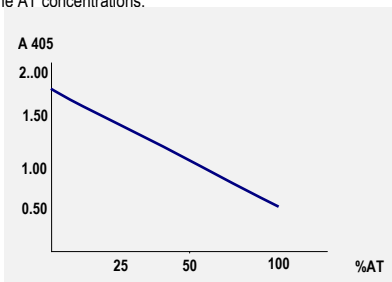
A new calibration curve must be carried out for each new lot of reagents, after each important maintenance of the analyzer, or when measured values for the quality controls are out of the acceptance range determined for the method. Each laboratory can establish its own acceptance ranges, according to the instruments and protocols used. Include at least one quality control (at different levels) in each test series.

RESULTS:

- For the end-point method, use a linear graph paper and plot on abscissae the AT concentrations (%) and on ordinates the corresponding absorbances (A405).
- Alternatively, statistics software can be used for establishing the dose response calibration curve. An inverse linear relationship is obtained between AT concentrations and Absorbances (A405).
- Draw the calibration curve obtained.
- Calculate the "r²" value. Calibration is acceptable if: **r² ≥ 0.98**, and if measured values for controls are in compliance.
- Usually, using the manual method, the A405 values range from about 1.80 (1.80 ± 0.30) for the 0% AT concentration, to about 0.50 (0.50 ± 0.30) for the 100% AT concentration. Indicatively, for the microplate method, A405 is expected from about 1.20 (1.20 ± 0.20) for 0%AT, to about 0.30 (0.30 ± 0.20) for 100% AT. A405 values can differ according to the instrument application used.
- The AT concentration in the tested sample (diluted 1:40) is directly obtained on the calibration curve (concentration corresponding to the measured A405). Results are expressed as % AT.
- When the kinetics mode is used, proceed the same way by plotting the ΔA_{405} values obtained, instead of A405.
- Using automated methods, the AT concentrations are directly calculated by the analyser, respectively to the calibration curve, and the sample dilution used.
- The dynamic range is from 0 to 100 % AT.

When the assay dilution is 1:40, the AT concentration is directly read on the calibration curve. When predilutions are used, multiply the measured AT concentration by the predilution factor in order to get the concentration in the tested specimen.

EXAMPLE OF CALIBRATION CURVE: The calibration curve below is an example only, using the water bath method. Only the calibration curve generated for the series of assays performed must be used for calculating the AT concentrations.



PERFORMANCE CHARACTERISTICS:

- **Dynamic range: 0 to 100 %** (ie 0 to 1IU/ml AT), linearity being verified in the 20-120% AT range.
- The detection threshold for the assay 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 minus 3 Standard Deviations (SD). This **detection threshold** is < 0.10 IU/ml, ie <10% AT.
- **Specificity:** An AT depleted plasma 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, but for research purposes only. 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 α_2 -macroglobulin) does not impact the assay. Plasmin, if present in the sample, is blocked by aprotinin present in R1.
- **Inter lots correlation (STAR):** N=73 Y = 1.01X + 1.04 r = 0.980 (Range 0-129% AT).
- Example of **reproducibility** results obtained using the STAR instrument (N=10).

	% AT	Intra Assay CV%	Inter Assay CV%
Normal Control	93	1.9	5.7
Abnormal Control	55	1.7	3.4

- The assay can also be performed on purified milieu, using corresponding appropriate calibration.

• Limitations of the procedure:

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. **x2 for 1:80**, or **x0.5 for 1:20**. In two-point kinetics methods, there is no significant interference for haemoglobin concentrations <5 mg/ml, bilirubin concentrations <0.6 mg/ml, and triglycerides <1.25 mg/ml, when added to normal plasma. These analytes can interfere in absorbance readings: in these cases, individual plasma blanks are necessary when end-point manual methods are used (acid stopped). Thrombin inhibitors (eg Hirudin, Argatroban) present in the tested sample may lead to overestimation of AT concentration. In order to get the optimal assay performances, the working instructions must be carefully observed. Each laboratory should verify performances in its exact working conditions.

The results obtained should be for research purposes only and not used for patient diagnosis or treatment.

REFERENCES:

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