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MEASUREMENT OF PROGRESSIVE ANTITHROMBIN (AT III) ACTIVITY

INTENDED USE:

Chromogenic assay for the quantitative determination of the “progressive” activity of Antithrombin (AT), heparin independent, in human citrated plasma using an anti Xa method, manual or automated.

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. In the absence of heparin, Antithrombin is a “slow acting”, progressive, inhibitor of coagulation serine esterases.

The assay is a chromogenic method based on the inhibition of Factor Xa, which is at a constant concentration and in excess, by Antithrombin(**without addition of heparin, but with a prolonged inhibition time between Factor Xa and tested AT III**). The remaining Factor Xa is then measured by its amycolytic 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.

As the assay is performed in the absence of heparin, it cannot be applied on plasmas from patients treated with Heparins (UFH or LMWH).

[AT.] + [Excess FXa] → [FXa-AT + [Remaining FXa]

[Remaining FXa] + SXa-11 → Peptide + pNA

Color development at 405 nm.

REAGENTS:

BIOPHEN AT (5) or BIOPHEN AT (2.5) kits, used without the corresponding R3 (reagent 3): Tris Buffer: kits used without the R3 buffer supplied in the kits; it must be replaced by the here below buffer, without Heparin.

RS: Special , Ready to use buffer: Tris Buffer, at pH 7.85, contains sodium azide (NaN₃). 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 (**RS**) 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.

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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 Antithrombin kits, and the specific buffer without heparin, 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 (test tube) or 4 ml (microplate)** (BIOPHEN AT (5)) or **2.5 ml (test tube) or 2ml (microplate)** (BIOPHEN AT(2.5)) of Tris Buffer (**RS**, without heparin). 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 (test tube) or 4 ml (microplate)** for BIOPHEN AT (5) or **2.5 ml (test tube) or 2ml (microplate)** for BIOPHEN AT (2.5) 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.

RS: SPECIAL Tris Buffer (without Heparin)

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. When open: 4 weeks at 2-8°C.

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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:

This progressive Antithrombin assay is designed for being used in chromogenic methods, automated (*provided that the instrument is able to manage the 1 hour incubation step used*), but it can also be used for end point manual methods. The assay is performed at the controlled temperature of 37°C and the colour development is measured at 405 nm. As the assay is a progressive measurement of ATIII, performed without heparin, the inhibition step is prolonged **and extended to 1 hour**.

CALIBRATION:

This chromogenic progressive AT III assay 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:

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% 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 (test tube) or 1:10 (microplate)** 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:	20 µL Plasma 1 :10	100 µL Plasma 1 :20
R1 : Facteur Xa preincubated at 37°C (reconst. according to the tube or microplate method)	80 µL	250 µL
Mix and Incubate for exactly 1 hour at 37°C, then introduce:		
R2 : Sxa-11 Substrate preincubated at 37°C (reconst. according to the tube or microplate method)	80 µL	250 µL
Mix and Incubate for 1 min at 37°C, exactly		
Stop the reaction by introducing:		
Citric Acid (20g/L)	80 µL	250 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.

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 progressive AT III chromogenic 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).

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LIMITATIONS OF THE PROCEDURE:

- There is no known drug interference in the assay, excepted the presence of Heparin or direct Factor Xa inhibitors.
- As the assay is an Anti-Xa method, there is no interference of Heparin Cofactor II, α 2-macroglobulin or α 1-Antitrypsin.
- 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 (indirect relationship)

- 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.

Calibration curve obtained for the progressive Antithrombin (AT III) activity (test tube):

