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BIOPHEN® AT anti-(h)-Xa LRT

Ref 221123

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

For in vitro diagnostic use only

Not for Sale in the US

Last revision: 28/04/2015

INTENDED USE:

BIOPHEN® AT anti-(h)-Xa LRT kit is a chromogenic assay for the in vitro quantitative determination of Antithrombin (AT) activity in human citrated plasma using an anti Xa method, manual or automated. Reagents of BIOPHEN® AT anti-(h)-Xa LRT kit are liquid, ready to use.
(LRT = Liquid Reagent Technology).

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. When complexed to heparin, Antithrombin becomes a potent and fast acting inhibitor of coagulation serine esterases.
BIOPHEN® AT anti-(h)-Xa LRT assay is a kinetics 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: Human Factor Xa
Human Factor Xa, liquid form, at pH about 7.85, containing heparin, bovine serum albumin (BSA) and sodium azide.
3 vials containing 3 ml of Factor Xa reagent.
R2: Reagent 2: FXa substrate
Chromogenic substrate, specific for Factor Xa (11-65), liquid form with stabilizers.
3 vials containing 3 ml of substrate reagent.

Note:
- Human Factor Xa was prepared from Human plasma, which was tested for the absence of infectious agents. However, no test may totally exclude the absence of infectious agents. As any product of Human origin, this factor Xa must be used with all the cautions required for handling a material potentially infectious.
- All the required cautions must be respected in order to avoid any risk of ingestion or accidental introduction of R1 or R2 in body. In case of skin contact, wash extensively with water. In case of contact with a wound, address to the appropriate medical service, and indicate the biological origin and the nature of the product.
- Human 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 are not interchangeable from lot to lot. Use only reagents from a same kit lot for testing AT.**

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:

Reagents:
- Distilled water, preferentially sterile.
- Acetic Acid (20%) or Citric Acid (2%) (End point method).
- Physiological saline (0.15M NaCl) or Imidazole Buffer (ref AR021)
- Plasma Calibrator, titrated for antithrombin activity (e.g. BIOPHEN® Plasma Calibrator Ref 222101) or equivalent
- Normal or Abnormal Control Plasmas, titrated for antithrombin activity (e.g. BIOPHEN® Normal Control Plasma Ref 223201, and BIOPHEN® Abnormal Control Plasma Ref 223301) or equivalent

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

STORAGE CONDITIONS:

BIOPHEN® AT anti-(h)-Xa LRT kit 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:

R1: Reagent 1: **Human Factor Xa**
- Ready to use.
- Let the reagent stabilize for 30 min at room temperature (18-25°C), before use, while shaking the vial from time to time.
- Homogenize well before each use, taking care of product viscosity, as well as when pipetting for manual method.

Stability of Factor Xa reagent, open, kept in its original vial:
- 5 weeks at 2-8°C.
- 7 days at room temperature (18-25°C).

R2 : Reagent 2: **Factor Xa specific Chromogenic substrate**
- Ready to use.
- Let the reagent stabilize for 30 min at room temperature (18-25°C), before use, while shaking the vial from time to time.
- Homogenize well before each use.

Stability of the substrate, open, kept in its original vial, and provided that any bacterial contamination or evaporation is avoided during use:
- 5 weeks at 2-8°C.
- 7 days at room temperature (18-25°C).

Cautions:
- In order to improve stability, reagents must be closed with their original screw cap following each use (white cap for FXa (R1), yellow cap for substrate (R2)).
- Reagents must be handled with care, in order to avoid any contamination during use.
- The substrate is slightly yellow. If the substrate becomes very yellow, this indicates the presence of a contaminant. It must be discarded, and a new vial must be used.
- Incubating the vials, for 30 min. at room temperature (18-25°C), allows stabilizing the reagents, and obtaining a homogeneous reactivity over time.
- In order to prevent evaporation of reagents, limit the liquid-air surface exchange.

Note:
- **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):

Preparation and storage of samples are performed as recommended by GEHT or NCCLS/CLSI.
- Samples:
  Human plasma collected on citrate anticoagulant where the activity of antithrombin must be measured.
- Collection:
  Blood (9 vol.) must be collected on trisodium citrate anticoagulant (1 vol.) through a net venipuncture. The first tube must be discarded. The delay between the collections and the tests is ideally 1 to 2 hours and should not exceed 4 hours.
- Centrifugation:
  Use a validated method in the laboratory to obtain a platelet-poor plasma, e.g., a minimum of 15 minutes at 2000 g at room temperature (18-25°C)
- Storage of plasma:
  - 4 hours at room temperature (18-25°C)
  - 2 months at -20°C.

TEST PROCEDURE:

BIOPHEN® AT anti-(h)-Xa LRT kit is designed for being used with kinetics methods, automated, but it can also be used for end point manual methods. Applications for the various analyzers are available upon request. The assay is performed at the controlled temperature of 37°C and the color development is measured at 405 nm.

CALIBRATION:

Calibration can be performed with BIOPHEN® Plasma Calibrator. Alternatively any commercially available plasma calibrator with a known AT concentration (C) or a normal pooled human citrated plasma (made with plasmas from at least 30 normal individuals, males or females, aged between 18 and 55 years, and out of any medication or disease), with the assigned value of **100%** AT. The assay includes a standard plasma dilution of **1:50**. By definition, this latter dilution of the pool represents the **100 %** AT activity. The dynamic range is from **10 to 150 %** AT. The **150 %** AT activity is then the **1:33.3** dilution of the **plasma pool** (in physiological saline).

The following calibration range can then be prepared as follows (from prediluted calibrator or pool):

% AT		Plasma Calibrator (µL)	Saline (µL)
0	**0**	0	500
12.5%	**C:8**	60	420
25%	**C:4**	125	375
50%	**C:2**	250	250
100%	**C**	500	0
150%	**3C/2**	Obtained by dilution factor : **33.3 x C:100 in saline**	

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

ASSAY PROTOCOL:

Manual Method:

Plastic tube: Dilute the tested samples and the controls **1:50** with physiological saline (0.15 M NaCl).

In a plastic tube preincubated at **37°C**, introduce:

Reagents	Test Tube
Diluted Calibrators, Controls or tested plasmas	200 µL
R1 : Factor Xa preincubated **at 37°C**	200 µL
Mix and Incubate at 37°C, for **1 minute exactly**, then introduce:	
R2: Substrate preincubated **at 37°C**	200 µL
Mix and Incubate at 37°C, for **1 minute exactly**	
Stop the reaction by introducing:	
Citric Acid (20g/L)	400 µL
Mix and measure the optical density at **405nm** against the sample blank.	

The yellow colour obtained is stable for at least 1 hour.

The sample blank is obtained by mixing the reagents in the opposite order from that of the test i.e.: Citric Acid (20 gl/L), substrate, diluted plasma, Factor Xa.

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

Automated methods:

Applications 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 specific application and cautions for each instrument.**

Note:
- If higher or lower reactive volumes are required for the method used, the 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 icteric, lipemic or hemolysed plasmas, or if the plasmas has a "colour" different from the usual one**.**

QUALITY CONTROL:

Using commercially available quality control plasmas, titrated for AT activity, 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: e.g. **BIOPHEN® Normal Control Plasma (#223201)** and **BIOPHEN® Abnormal Control Plasma (#223301).**

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. At least one quality control (at different levels) should be included in each test series.

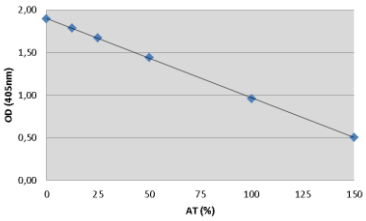
RESULTS:

- For the end point method, using a **linear** graph paper, plot on ordinates the corresponding absorbance (**A405**) and on abscissae the AT concentration (**%**). 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).
- Calibration is acceptable if: **$r^2 \geq 0.98$**, and if measured values for controls are in compliance.
- Usually, using the manual method (test tube), the A405 value for the 0 %AT concentration is of about 1.7% ± 0.3. A405 values can differ according to the instrument application used.
- The AT concentration in the tested sample is directly obtained on the calibration curve. Results are expressed as % of AT.
- Using automated methods, the AT concentrations are directly calculated by the analyzer, respectively to the calibration curve.
- The dynamic range is from **10 to 150 %;** the assay being linear up to 150% AT activity.

When the assay dilution is **1:50**, the AT concentration is directly read on the calibration curve. When different dilutions are used, the results must be multiplied by the dilution factor "**D**", divided by **50** i.e. **D:50.**

EXAMPLE OF CALIBRATION CURVE:

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



PERFORMANCES AND CHARACTERISTICS:

- Dynamic range **10-150%** in assayed plasma.
- The detection threshold is calculated by measuring the "apparent" A405 obtained for an Antithrombin deficient sample less 2 standard deviations (SD). This detection threshold is **≤ 10%.**
- Specificity: AT poor plasma was measured ≤15%.
- Example of performances obtained for samples with variable Antithrombin concentrations using Sysmex® CS-2000i analyzer:

	Intra Assay			
	N	Mean	SD	CV(%)
Sample 1	10	95.9	1.0	1.0
Sample 2	10	32.4	0.8	2.5

- The BIOPHEN® AT anti-(h)-Xa LRT assay shows good correlation with Siemens INNOVANCE® AT reagent on Sysmex® CS-2000i analyzer:
  n=59 range 17 to 150% AT Correlation r=0.998

LIMITATIONS OF THE PROCEDURE:

- As the assay is an Anti-Xa method, there is no expected interference of Heparin Cofactor II, ∝2-macroglobulin or ∝1-Antitrypsin.
- No significant interference was noticed using the Sysmex® CS instrument method for haemoglobin concentrations up to 500 mg/dl, bilirubin concentrations up to 28 mg/dl, triglycerides up to 300 mg/dl, and heparin up to 4 IU/mL by spiking experiment in plasma. Some analytes can interfere in absorbance readings: in these cases, individual plasma blanks are necessary when end-point manual methods are used.
- Direct Factor Xa Inhibitors, such as Rivaroxaban, Apixaban, etc…, may induce an overestimation of measured AT in patients treated with these drugs.
- In order to get the optimal performances of the assay, the procedural instructions must be strictly respected.

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.

Normal range expected about 75% up to about 125%, to be verified and validated in the exact laboratory working conditions (instrument and application, lot, calibrator used…).

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.

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.

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