LIAPHEN AT
A120002-RUO

Turbidimetric latex immunoassay for the quantitative determination of Antithrombin (AT)

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

Last revision: 28/09/2011

INTENDED USE:

LIAPHEN AT kit is a latex immunoassay for measuring Antithrombin (AT) in human citrated plasma, using a manual or an automated method, exclusively in vitro. **This kit is for research use only and should not be used for patient diagnosis or treatment.**

SPECIMEN:

Human citrated plasma.

ASSAY PRINCIPLE:

LIAPHEN AT is an immuno-turbidimetric assay using latex particles, for the in vitro determination of Antithrombin (AT). When the tested specimen is mixed with the reaction buffer (**R2**) and the latex reagent (**R1**), the anti-AT antibodies coupled onto latex particles react with AT present in the sample and agglutination occurs. The amount of agglutination is directly proportional to the amount of AT in the sample and is measured by light absorption (A620nm or specific automate wavelength).

Latex + AT ⟶ Aggregates (A620nm,…)

REAGENTS:

R1: Reagent 1: Latex reagent
Latex microparticles coated with polyclonal goat anti-(h)-AT antibodies purified by affinity chromatography. Ready to use liquid reagent.
2 vials of 2 ml

R2: Reagent 2: Reaction buffer
Hepes NaCl Buffer, containing 1% BSA, and 0.9g/L sodium azide as preservative. Ready to use.
2 vials of 10 mL.

Warning:
- Bovine Serum Albumin (BSA) was 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.
- Sodium azide (0.9 g/l) 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:

- Physiological saline (NaCl 9g/L)
- Plasma Calibrator titrated for AT *(***ex: BIOPHEN Plasma Calibrator #A222101, HYPHEN BioMed).**
- **Or** Reference material for AT:Ag (international or internal).
- Normal and Abnormal Quality Control Plasmas *(***ex: BIOPHEN Normal Control Plasma #A223201, and BIOPHEN Abnormal Control Plasma #A223301)** titrated for AT.

Material:

- Spectrophotometer, with a wave-length set up at 620 nm, photometer or automates for chromogenic assays.
- Stop watch.
- Calibrated pipettes.
- Cuvette or microplate.

TRACEABILITY TO THE REFERENCE MATERIAL:

Internal reference standard for AT:Ag concentration was established against the SSC/ISTH secondary coagulation standard lot #3 from NIBSC, and the 2nd International Standard AT, plasma, from NIBSC, code 93/768.

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.

<u>Note</u>: 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: Latex reagent
- Let to homogenize for 30 minutes at room temperature (18-25°C).
- Mix gently before each use.

Stability of opened reagent R1, kept in its original hermetically closed vial, provided any evaporation or contamination is avoided:
- 7 days at room temperature (18-25°C)
- 6 months at 2-8°C

R2: Reagent 2: Reaction Buffer
Ready to use buffer.
- Let to homogenize for 30 minutes at room temperature (18-25°C) prior to use.
- Mix gently before each use.

Stability of opened buffer R2, kept in its original hermetically closed vial, provided any evaporation or contamination is avoided:
- 7 days at room temperature (18-25°C)
- 6 months at 2-8°C

Cautions:
- Reagents must be handled with care, in order to avoid any contamination during use.
- After use, vials should be closed with their specific caps.

Note:
- 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.**

TESTED SPECIMEN:

Human citrated plasma.
Plasma preparation: Blood (9 vol.) must be collected on 0.109M citrate anticoagulant (1 vol.); plasma supernatant is decanted following a 20 min. centrifugation at 2,500 g.
Storage: citrated plasma should be tested within 8 hours or stored frozen at –20°C or colder for up to 6 months, and thawed for 15 min. at 37°C just before use.

Note: Refer to GEHT or CLSI recommendations for further instructions on specimen collection, handling and storage. Discard any plasma presenting an unusual aspect (haemolysed, lipaemic aspect….).

TEST PROCEDURE:

LIAPHEN AT kit is designed for being used with automated kinetic methods but it can also be used with the manual method. Adaptations to the various automates are available upon request.

Using the manual method, the assay is performed at the controlled temperature of **37°C** and the agglutination development is measured at **620 nm** (other wavelengths can be used, preferentially between 450 and 700nm).

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 **<u>1:15</u>**. By definition, this latter dilution of the pool represents the **100 % AT**. The dynamic range is from **0 to 150 %** AT. The 150 **%** AT is then the **1:10** dilution of the **plasma pool** (in physiological saline).

- **Or** the kit can be calibrated with a commercially available plasma calibrator, titrated for AT concentration (e.g. **Biophen Plasma Calibrator #A222101**).
If calibration is performed with a commercially available plasma calibrator, with a known AT concentration (**C**): the **150%** calibration point**,** with a working dilution at **1:15** (manual method)**,** is obtained by diluting the calibrator using the following dilution factor **D = 10*C:100, i.e. <u>D = C (in %) : 10</u>**.

Prepare **2 ml** of the **1:10** dilution of the normal plasma pool, or of the **1:D** (**D=C:10)** dilution of the AT standard. This corresponds to 150% AT (noted **C1**); the calibration curve can then be obtained by preparing serial dilutions as follows:

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Standard	C1	C2	C3	C4	C5	C6
% AT	**150**	**100**	**75**	**50**	**25**	**0**
Vol of AT standard	2000µL	600µL of **C1**	500µL of **C1**	500µL of **C2**	500µL of **C4**	0µl
Vol of Physiological saline	0 µL	300µL	500µL	500µL	500µL	1000µL

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

TESTED SAMPLES AND CONTROLS:

Plasma samples and controls are assayed at the **1:15** (**manual method**) dilution in physiological saline. For purified AT the appropriate dilution must be done in physiological saline with 1% of BSA (expected final concentration in the range 0.5 to 10µg/ml AT). The diluted samples must be tested within 2 hours.

ASSAY PROTOCOL:

- ***Manual Method***

Prepare just before use the appropriate volume of latex R1 diluted 1:5 in the reaction buffer (R2).	
Reagents	**Manual method**
Calibrators, or diluted tested plasmas, or Controls	100 µl
R1 diluted 1:5 in R2 preincubated **at 37°C and homogenized before use.**	400 µl
Mix and incubate for **15 min at 37°C exactly** and, immediately after:	
Mix and read the Absorbance at **620nm** against physiological saline. **Respect the same overall reaction time for each sample.**	

Cautions:
- **Using manual method, a calibration curve must be performed for each test series.**
- Run a sample blank in presence of highly lipemic, icteric or haemolysed plasmas, or if the plasmas has a different "colour" from the standard.

- ***Automated methods:***

Manual dilution of the latex is not necessary for automated methods, the two reagents are loaded on the instrument. Adaptations to the various analyzers are available upon request. The assay is then performed kinetically. Sample blanks are automatically subtracted. The working dilution and the volume of reagents in the test may vary depending on the instrument, **refer to the specific adaptation and specific cautions for each instrument.**

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), Hyphen BioMed.** Each laboratory should verify its own target value and acceptance range, in the exact working conditions, for each new lot of controls.

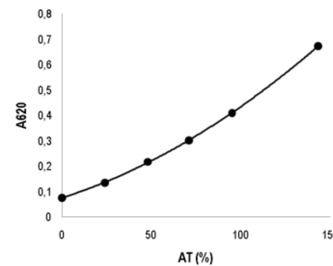
Note :
- A new calibration curve must be carried out better with each series, and for each new lot of reagents, after each important maintenance of the analyzer, or when measured values for the quality controls are out of the acceptance range determined for the method.
- Each laboratory can establish its own acceptance ranges, according to the instruments and protocols used.

RESULTS:

- For the manual method, plot on abscissae the AT concentration (%) and on ordinates the corresponding absorbance (**A620nm**) and draw the calibration curve. Alternatively use a software to draw the calibration curve that best fits your values (3rd order polynomial, spline curve,…). Calibration is validated when controls are measured within their acceptance range and $r^2 \geq 0.98$.
  The AT concentration in the tested sample (diluted **1:15** using the manual method) is directly obtained on the calibration curve. Results are expressed as % of AT.
- Using the **manual method**, when the assay dilution is **1:15**, the AT concentration is directly read on the calibration curve. When other predilutions are used, multiply the measured AT concentration by the complementary predilution factor in order to get the concentration in the tested specimen.

EXAMPLE OF CALIBRATION CURVE:

The calibration curve below is an example only, and is obtained with the manual method. Only the calibration curve generated for the series of assays performed must be used for calculating the concentrations.



PERFORMANCE CHARACTERISTICS:

- **Dynamic range**: using manual method, from 0 to 150 % of AT.
- **Limit of Quantification:** 11.4%, defined as the lowest concentration giving a CV< 20% and Bias < 20% on N=16 replicates, collected over 4 days, with 4 replicates per day.
- **Specificity**: no reactivity with AT deficient plasma (<5% of AT).
- **Precision**: 3 plasmas with high, medium and low AT concentrations have been assayed during 8 days, for a total of 10 runs. Results are presented in the table below:

	AT concentration (%)	Within-run repeatability	Total precision
Level 1	102.0	1.9%	4.4%
Level 2	63.3	1.5%	4.0%
Level 3	22.7	3.0%	7.4%

- **High-dose hook effect:** No hook effect is observed for AT concentrations below 200%.
- **Interferences:**
  - No interference is observed for: Unfractionated Heparin (UFH) and Low Molecular Weight Heparin (LMWH) ≤ 2 IU/ml, Bilirubin ≤ 0.2 g/L, Haemoglobin ≤ 2 g/L, Intralipid® ≤ 0.75% (corresponding to 20 g/L of triglycerides).
  - The presence of rheumatoid factor may result in an overestimation of AT concentration.
  - **The results obtained should be for research purposes only and not used for patient diagnosis or treatment.**

GENERAL INFORMATION AND BIOCHEMISTRY:

Antithrombin is a single chain glycoprotein synthesized in the liver and has a molecular weight of about 58 200 daltons (2). Its concentration in normal plasma is about 125µg/ml. AT is a progressive inhibitor of thrombin (factor IIa) but also of other serine proteases, such as factor Xa, factor IXa, XIa and XIIa, plasmin and kallikrein. Heparin accelerates dramatically the inhibition of factor IIa and Xa (1). Antithrombin concentration, like protein C and protein S concentrations, plays an important role in the hemostatic balance.

REFERENCES:

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4. Tollefsen D.M. Laboratory Diagnosis of Antithrombin and Heparin Cofactor II deficiency. Seminars in *Thromb haemost 16, 162-168 (1990).*

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