Antithrombin 2.5 kit is a chromogenic assay for the quantitative determination of the heparin cofactor activity of Antithrombin (AT) in human citrated plasma, using an anti Xa method, manual or automated.

**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.

**PREPARATION:**

**STORAGE CONDITIONS**

**REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED**

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**REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED**

**STABILITY OF REGENERATED ANTITHROMBIN**

**PREPARATION OF PLASMA (SPECIMEN COLLECTION):**

**TEST PROCEDURE:**

**CALIBRATION:**

<table>
<thead>
<tr>
<th>% AT</th>
<th>Plasma Calibrator (µl)</th>
<th>Physiological Saline (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>C/4</td>
<td>125</td>
<td>375</td>
</tr>
<tr>
<td>C/2</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>C</td>
<td>500</td>
<td>0</td>
</tr>
</tbody>
</table>
**ASSAY PROTOCOL:**

**Manual Method:**
Dilute the tested samples, the controls and the calibration solutions 1:20 with physiological saline (0.15 M Sodium Chloride).

In a microplate well, or in a plastic tube preincubated at 37°C, introduce:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Microplate</th>
<th>Test Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrators, Controls or tested plasma, diluted 1:20</td>
<td>40 µL</td>
<td>80 µL</td>
</tr>
<tr>
<td>R1: Factor Xa preincubated at 37°C</td>
<td>100 µL</td>
<td>200 µL</td>
</tr>
</tbody>
</table>

Mix and Incubate for 1 min at 37°C, then introduce:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Microplate</th>
<th>Test Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>R2: S-Xa-11 Substrate preincubated at 37°C</td>
<td>100 µL</td>
<td>200 µL</td>
</tr>
</tbody>
</table>

Mix and Incubate for 1 min at 37°C, exactly.

Stop the reaction by introducing:

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Microplate</th>
<th>Test Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric Acid (20g/L)</td>
<td>100 µL</td>
<td>200 µL</td>
</tr>
</tbody>
</table>

Mix and measure the optical density at 405 nm 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 µL), S-Xa-11 substrate, diluted plasma, Factor Xa.

**Automated methods:**
Adaptations to the various analysers (STA-R, BCT, BCS, etc.) are available upon request. Reconstitution volumes can vary according to the automate used. 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.
- Run a sample blank in presence of highly lipemic, icteric or haemolysed plasma.

**QUALITY CONTROL:**
To use of quality control plasma allows validating the calibration curve, as well as the homogeneity reactivity of the BIOPHEN Antithrombin 2.5 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 223201)
- **BIOPHEN Abnormal Control Plasma:** (ref 223301).

**LIMITATIONS OF THE PROCEDURE:**
- 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, α2-macroglobulin or α1-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 hyperlipemic patients. These analyses 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 abscissa, the Antithrombin concentration (%) and on ordinate the corresponding absorbance (A405).
- The Antithrombin concentration in the tested sample is directly obtained on the calibration curve. 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.

**VALIDATION OF CALIBRATION CURVE:**
The calibration curve is acceptable when the concentrations measured for the Control Plasmas are within the acceptance range.

**PERFORMANCES AND CHARACTERISTICS:**
- 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%.
- Example of Intra-Assay and Inter-Assay reproducibilities obtained for samples with variable Antithrombin concentrations (ACL):

<table>
<thead>
<tr>
<th>Samples</th>
<th>AT concentrations %</th>
<th>Intra-Assay CV%</th>
<th>N</th>
<th>Inter-Assay CV%</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1</td>
<td>109</td>
<td>0.73</td>
<td>10</td>
<td>2.57</td>
<td>12</td>
</tr>
<tr>
<td>Sample 2</td>
<td>69</td>
<td>0.66</td>
<td>10</td>
<td>2.49</td>
<td>12</td>
</tr>
<tr>
<td>Sample 3</td>
<td>51</td>
<td>0.92</td>
<td>10</td>
<td>3.72</td>
<td>12</td>
</tr>
</tbody>
</table>

**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 405 nm, and the level of AT is linear in the 80-120% range of normal plasma.

**CLINICAL INFORMATION:**
Sporadically thrombotic/bloembolic diseases are observed in presence of congenital deficiencies. These congenital deficiencies are classified in 4 different groups:
- **Type I** Decreased Antithrombin concentration and decreased Antithrombin activity; this is the most frequent case.
- **Type II R5 (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.

**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 2.5 mL of Tri-Buffer, without heparin [Reference ARTA: AT-Tri-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 (0.750.30/AT-prog/Anti Xa).

In presence of the HBS variant, the patient has a normal Antithrombin activity with this method.

**REFERENCES:**