BIOPHEN™ ANTI-IIa (2 Stages Heparin Assay)  
Ref 220005  
(R1, R2, R3: 2 x 1 mL)  

Two stages chromogenic method for the Heparin anti-IIa activity measurement on plasma or purified medium, according to Pharmacopoeia (USP, EP).

**INTENDED USE:**
This BIOPHEN™ ANTI-IIa (2 Stages Heparin Assay) kit is a two-stage chromogenic assay for measuring the activity of heparin (UFH or LMWH), in manual or automatic method. This method is proposed only to test heparin in human citrated plasma, or in purified solution. This kit is for research use only and must not be used for patient diagnosis or treatment.

**SUMMARY AND EXPLANATION:**
Heparin is a sulfated polysaccharide with a high affinity for antithrombin. When complexed with antithrombin, heparin inhibits efficiently thrombin and also the other serine proteases. Anti-IIa assays are the right methods for measuring the anti-thrombin activity of large heparin molecules. This heparin assay is a two-stage Anti-IIa assay for measuring accurately and sensitively heparin concentrations in plasma or in purified systems. Tested sample need to be diluted before assay by it.

**PRINCIPLE:**
The BIOPHEN™ ANTI-IIa (2 Stages Heparin Assay) method is a two stage method based on the inhibition of a constant amount of Thrombin (IIa), by the tested heparin in presence of exogenous antithrombin (stage 1), then hydrolysis of a Thrombin specific chromogenic substrate (CS-O138), by the residual Thrombin in excess (stage 2), pNA is then released from the substrate. The amount of pNA released (measured at 405 nm) is then a relation of the residual Thrombin activity. There is an inverse relationship between the concentration of heparin and color development.

**REAGENTS:**
R1: Reagent 1 : ATIII (h)
- Human Antithrombin (ATIII), lyophilized vial containing about 1.25 IU/mL. Contains BSA.
- 2 vials of 1 mL.

R2: Reagent 2 : Human Thrombin
- Purified human Thrombin, mainly in the α form, lyophilized vial containing about 120 NIH (or IU), or about 150 nits (when determined in optimized conditions with CS-O138 specific substrate).
- 2 vials of 1 mL.

R3: Reagent 3 : Thrombin specific chromogenic substrate
- Chromogenic substrate specific for Thrombin (CS-O138), vial of about 6.25 µmol, lyophilized in presence of mannitol.
- 2 vials of 1 mL.

**WARNINGS AND PRECAUTIONS:**
- Biological products must be handled with all necessary precautions and considered as being potentially infectious.
- A yellow color indicates a contaminated substrate. Discard the vial and use a new one.
- Waste should be disposed of in accordance with applicable local regulations.
- Use only the reagents from the same batch of kits. Do not mix reagents from different kit batches when performing an assay; they are optimized for each batch of kits.
- Handle the reagents with care to avoid contamination during use. If possible, avoid reagent evaporation during use by limiting the liquid-air exchange surface. Evaporation reduces the reagent stability in the vials.
- The human plasma used to prepare the human thrombin has been tested by recorded methods and is certified free of HIV antibodies, Hbs Antigen and HCV antibodies. The bovine plasma used to prepare the BSA has been tested by recorded methods and is certified free of infectious agents, in particular the causative agent of bovine spongiform encephalopathy.
- To preserve reagent stability, seal the vials after use with their respective caps.
- Aging studies, conducted over a 3-week period at 30°C, show that the reagents can be used up to 60 days at room temperature over a short period of time, without degradation.
- Create a plasma blank if this latter is icteric, lipaemic, haemolyzed, or if its color differs from the standard plasma.
- When employing the kinetic method, use AOD 405 instead of OD 405.
- α-Thrombin has a high clotting activity respectively to other and more degraded human thrombin preparations, for a same chromogenic activity. NIH is a clotting unit. Thrombin concentration is exactly adjusted from lot to lot for offering an optimized assay reactivity and linearity.
- For in vitro use.

R2: H315: Causes skin irritation.  
H319: Causes serious eye irritation.

**REAGENT PREPARATION AND STABILITY:**
The reagents are lyophilized under a vacuum in their vials. To avoid any product loss when opening the vial of lyophilized reagents, gently remove the freeze-drying stopper.

**R1:** Reagent 1: ATIII (h)
Reconstitute the contents of each vial with exactly 1 mL distilled water, shake vigorously until fully dissolved. Allow to stabilize for 30 min. at room temperature (18-25°C), shaking occasionally.

- Just before use, dilute 1/5 in the appropriate buffer according to the Heparin to be assayed (see table below, if the whole vial is used, add 4 mL of buffer to the 1 mL of reconstituted ATIII).
- Homogenize the reagent prior to use.
- Reagent stability after reconstitution, excluding any contamination or evaporation, and stored in the original vial, is of:
  - 15 days at 2-8°C,  
  - 4 days at room temperature (18-25°C),  
  - 6 months frozen at -20°C or less*

**R2:** Reagent 2: Human Thrombin
Reconstitute the contents of each vial with exactly 1 mL distilled water, shake vigorously until fully dissolved. Allow to stabilize for 30 min. at room temperature (18-25°C), shaking occasionally.

- Just before use, dilute 1/5 in the appropriate buffer according to the Heparin to be assayed (see table below, if the whole vial is used, add 4 mL of buffer to the 1 mL of reconstituted Thrombin).
- Homogenize the reagent prior to use.
- Reagent stability after reconstitution, excluding any contamination or evaporation, and stored in the original vial, is of:
  - 15 days at 2-8°C,  
  - 4 days at room temperature (18-25°C),  
  - 6 months frozen at -20°C or less*

**R3:** Reagent 3: Thrombin specific chromogenic substrate
Reconstitute the contents of each vial with exactly 1 mL distilled water, shake vigorously until fully dissolved. Allow to stabilize for 30 min. at room temperature (18-25°C), shaking occasionally.

- Just before use, dilute 1/5 in the appropriate buffer according to the Heparin to be assayed (see table below, if the whole vial is used, add 4 mL of buffer to the 1 mL of reconstituted substrate).
- Homogenize the reagent prior to use.
- Reagent stability after reconstitution, excluding any contamination or evaporation, and stored in the original vial, is of:
  - 15 days at 2-8°C,  
  - 4 days at room temperature (18-25°C),  
  - 6 months frozen at -20°C or less*

*Thaw only once, as rapidly as possible at 37°C, adapting the incubation period to the volume of reagent. The stability of the thawed reagent should be checked under laboratory work conditions.

**STABILITY OF DILUTED REAGENTS:**
Stability of diluted reagents should be checked in the working conditions of the laboratory user.

**STORAGE CONDITIONS:**
Unopened reagents should be stored at 2-8°C in their original packaging. Under these conditions, they can be used until the expiry date printed on the kit.

**REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED:**
- Reagents:
  - Distilled water.
  - 20% acetic acid or 2% citric acid (and point method).
  - Specific buffers such as:

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-EDTA/NaCl, pH 8.40</td>
<td>AP003K</td>
</tr>
<tr>
<td>Tris-EDTA/NaCl, pH 7.40</td>
<td>AP002K</td>
</tr>
<tr>
<td>Tris-EDTA/NaCl, pH 6.40</td>
<td>AP001K</td>
</tr>
</tbody>
</table>

**Calibrators and controls with known titration for Heparin to be assayed.**

**For plasma assay, it is possible to use following calibrator and control:**

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOPHEN UFH Control Plasma*</td>
<td>223101-R0D</td>
</tr>
<tr>
<td>BIOPHEN UFH Calibrator*</td>
<td>222301-R0D</td>
</tr>
</tbody>
</table>

*Calibrators titrated in NIH activity.

**INTERNATIONAL REFERENCE:**
Compliant with the pharmacopoeia used or internal reference material, specific for heparin to measure.

**HYPHEN BioMed**
95000 Neuville sur Oise - FRANCE

**D750-02/B1/0005/v2**
Materials:
- Spectrophotometer or automatic instrument for chromogenic assays.
- Stopwatch; Calibrated pipettes; Plastic tubes or microplate.

SPECIMEN COLLECTION AND PREPARATION:
Specimens should be prepared and stored in accordance with applicable local guidelines.
- Specimens:
  Human plasma obtained from anticoagulated blood (trisodium citrate).

Collection:
The blood (19 volumes) should be carefully collected onto the trisodium citrate anticoagulant (1 volume) (0.109 M) by clean venipuncture. Specific collection tubes for unfractionated heparin testing, such as the CTAD (Citrate, Threophyline, Adenosine and Dipyridamole) tubes, can be used. Discard the first tube.

Centrifugation:
Because of the potential for heparin neutralization by platelet factor 4, time before centrifugation should not exceed 1 hour at room temperature for specimen collected in sodium citrate and 4 hours for CTAD.

Use a validated method in the laboratory to obtain platelet-poor plasma, e.g., a minimum of 15 minutes at 2500g at room temperature (18-25°C) and plasma must be decanted into a plastic tube.
- Plasma storage:
  o 4 hours at room temperature (18-25°C).
  o 1 month at -20°C.
  o 18 months at -70°C.

Frozen plasma specimens should be thawed rapidly at 37°C, then shaken thoroughly and tested immediately. Resuspend any precipitate by shaking vigorously immediately after thawing and before use.

PROCEDURE:
The kit can be used for kinetic, automated or manual (endpoint) methods. Perform the test at 37°C and read color intensity at 405nm.

Automated methods:
Applications for the various analyzers are available on request. See the specific application and specific precautions for each analyzer.

ASSAY METHOD:
1. Reconstitute the calibrators and controls (same matrix as sample) as indicated in the specific instructions. Calibrators should be diluted using specific buffer, according to Heparin type to be measured, as described in the table below in order to establish the calibration range:

<table>
<thead>
<tr>
<th>Concentration (IU/mL)</th>
<th>LMWH solution at 11µL</th>
<th>Specific buffer</th>
<th>Dilution</th>
<th>Specific Buffer</th>
<th>Calibrators</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.135</td>
<td>135µL</td>
<td>250µL</td>
<td>1/25</td>
<td>AR005L (EP)</td>
<td>1.25</td>
</tr>
<tr>
<td>0.25</td>
<td>135µL</td>
<td>500µL</td>
<td>1/50</td>
<td>AR005L (EP)</td>
<td>1.25</td>
</tr>
<tr>
<td>0.50</td>
<td>135µL</td>
<td>750µL</td>
<td>1/100</td>
<td>AR005L (EP)</td>
<td>1.25</td>
</tr>
<tr>
<td>0.75</td>
<td>135µL</td>
<td>1mL</td>
<td>1/25</td>
<td>AR005L (EP)</td>
<td>1.25</td>
</tr>
<tr>
<td>1.00</td>
<td>135µL</td>
<td>250µL</td>
<td>1/25</td>
<td>AR005L (EP)</td>
<td>1.25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration (IU/mL)</th>
<th>UFH solution at 11µL</th>
<th>Specific buffer</th>
<th>Dilution</th>
<th>Specific Buffer</th>
<th>Calibrators</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.135</td>
<td>135µL</td>
<td>250µL</td>
<td>1/25</td>
<td>AR028K (USP)</td>
<td>1.25</td>
</tr>
<tr>
<td>0.25</td>
<td>135µL</td>
<td>500µL</td>
<td>1/50</td>
<td>AR028K (USP)</td>
<td>1.25</td>
</tr>
<tr>
<td>0.50</td>
<td>135µL</td>
<td>750µL</td>
<td>1/100</td>
<td>AR028K (USP)</td>
<td>1.25</td>
</tr>
<tr>
<td>0.75</td>
<td>135µL</td>
<td>1mL</td>
<td>1/25</td>
<td>AR028K (USP)</td>
<td>1.25</td>
</tr>
<tr>
<td>1.00</td>
<td>135µL</td>
<td>250µL</td>
<td>1/25</td>
<td>AR028K (USP)</td>
<td>1.25</td>
</tr>
</tbody>
</table>

   For the plasma, it is possible to use calibrators available (i.e. BIOPHEN UFH Calibrator 222301-RUO)

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

2. Dilute the samples and controls in specific buffer, as described in the table below:

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Reference</th>
<th>Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMWH control plasma</td>
<td>n.a.</td>
<td>AR005L (EP)</td>
</tr>
<tr>
<td>BIOPHEN UFH control plasma</td>
<td>223301-RUO</td>
<td>AR028K (USP)</td>
</tr>
<tr>
<td>LMWH samples</td>
<td>n.a.</td>
<td>AR005L (EP)</td>
</tr>
<tr>
<td>UFH samples</td>
<td>n.a.</td>
<td>AR028K (USP)</td>
</tr>
</tbody>
</table>

   Establish the calibration curve and test it with the quality controls. If stored at room temperature (18-25°C), test the diluted specimens within 2 hours. The exact calibrator and control concentrations for each batch are indicated on the flyer provided with the kit.

3. Dispense the following to the wells of a microplate, or to a plastic tube incubated at 37°C:

<table>
<thead>
<tr>
<th>Microplate</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample, calibrator or control diluted</td>
<td>40 µL</td>
</tr>
<tr>
<td>R1: Human Antithrombin Precipitated at 37°C</td>
<td>40 µL</td>
</tr>
<tr>
<td>Mix and incubate at 37°C for 2 minutes, then introduce:</td>
<td>200 µL</td>
</tr>
<tr>
<td>R2: Human Thrombin Precipitated at 37°C</td>
<td>40 µL</td>
</tr>
<tr>
<td>Mix and incubate at 37°C for exactly 2 minutes, then introduce:</td>
<td>200 µL</td>
</tr>
<tr>
<td>R3: Substrate Precipitated at 37°C</td>
<td>200 µL</td>
</tr>
<tr>
<td>Mix and incubate at 37°C for exactly:</td>
<td>90 sec</td>
</tr>
<tr>
<td>Stop the reaction by introducing:</td>
<td></td>
</tr>
<tr>
<td>Citric acid (2%)</td>
<td>80 µL</td>
</tr>
</tbody>
</table>
   | Mix and measure the absorbance at 405 nm against the corresponding blank.

Or acetate acid (1%). The yellow color is stable for 2 hours.

The sample blank is obtained by mixing the reagents in the reverse order to that of the test: Citric acid (2%), R3, R2, R1, dilute sample.

Measure the optical density at 405 nm. Subtract the measured blank value from the absorbance measured for the corresponding test.

If a reaction volume other than that specified above is required for the method used, the ratio of volumes must be strictly observed to guarantee assay performance. The user is responsible for validating any changes and their impact on all results.

QUALITY CONTROL:
The use of quality controls serves to validate method compliance, along with between-test assay homogeneity for a given batch of reagents. Include the quality controls with each series, as per good laboratory practice, in order to validate the test. A new calibration curve should be defined, preferably for each test series, and at least for each new reagent batch, or after analyzer maintenance, or when the measured quality control values fall outside the acceptable range for the method.

Each laboratory must define its acceptable ranges and verify the expected performance in its analytical system.

RESULTS:
- For the manual endpoint method, plot the calibration curve on a semi-logarithmic graph paper plot, with the OD 405 nm (log) along the Y-axis and the Heparin concentration, expressed as IU/mL along the X-axis.
- The concentration of Heparin in the test specimen is directly inferred from the calibration curve, if the standard dilution is used.
- Results are expressed in IU/mL.
- Multiply the concentration measured by the dilution factor used (i.e. x25 for plasma).

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

LIMITATIONS:
- To ensure optimum test performance and to meet the specifications, the technical instructions validated by BIOPHEN BioMed should be followed carefully. The laboratory is responsible for validating any changes made to these instructions for use.
- Any reagent presenting an unusual appearance or showing signs of contamination must be rejected.
- Any suspicious samples or those showing signs of activation must be rejected.
- Any plasma displaying a coagulum or showing signs of contamination must be rejected.
- If a higher working range for heparin is required, the standard assay dilution (d=1:25) can be adjusted accordingly. For example, use a 1:50 dilution (i.e. d:2) for a working range from 0 to 2 IU/mL or a 1:100 dilution (i.e. d:4) for a working range from 0 to 4 IU/mL in the tested specimen. The heparin concentrations measured must be multiplied by the dilution factor.
- Volumes and incubation times have been harmonized for easier handling and automation of the method, but are consistent with the reactional concentration recommended by Pharmacopoeia.
- The dilution buffer for LMWH protocol (AR028K) doesn’t contain a carrier molecule anhydrous USP. At very high dilution, the addition of the carrier molecule (BSA type) is likely to improve the robustness of the results.

REFERENCES:
2. USP40, effective May 1, 2017.

SYMBOLS:
Symbols used and signs listed in the ISO 15223-1 standard, see Symbol definitions document.

Changes compared to the previous version.