INTENDED USE:
The BIOPHEN Heparin 3 kit is a chromogenic assay for the quantitative determination of Unfractionated Heparin (UFH) in human citrated plasma using automated or manual method.

SUMMARY AND EXPLANATION:
Unfractionated Heparin (UFH) is currently used for curative or preventive indications. Measuring the heparin concentration in patients’ plasma allows monitoring the therapy and adjusting drug dosage.

PRINCIPLE:
BIOPHEN Heparin 3 is a chromogenic anti-Xa method developed for measuring homogeneously heparin (UFH), in plasma. Heparin is a sulphated polysaccharide with a high affinity for antithrombin (AT). When complexed with heparin, antithrombin exhibits a fast acting and potent inhibitory activity for coagulant serine esterases: IXa, Xa and thrombin.

Anti-Xa assays are then the methods of choice for measuring heparins and their analogues. The BIOPHEN Heparin 3 assay is a kinetic method based on the inhibition of a constant and in excess amount of Factor Xa, by heparin to be assayed, in the presence of endogenous antithrombin. The residual factor Xa hydrolyzes a specific chromogenic substrate (SXA-11) releasing paranitroaniline (pNA). The quantity of released pNA (measured by absorbance at 405 nm) is inversely proportional to the concentration of heparin present in the medium reaction.

REAGENTS:
Reconstitution volume has to be adjusted according to the analyzer used. Refer to specific application guide for each analyzer.

R1:
Reagent 1: Chromogenic substrate specific for Factor Xa (SXA-11), lyophilized in presence of mannitol.
3 vials of 3 mL (about 7.5 mg/vial).

R2:
Reagent 2: Bovine Factor Xa, Lyophilized. Contains Dextran Sulfate.
3 vials of 3 mL (about 7.5 g/vial).
Reagent R2 contains small amounts of sodium azide (0.9 g/L) and dextran sulfate, see WARNINGS AND PRECAUTIONS.

WARNINGS AND PRECAUTIONS:
- Biological products must be handled with all necessary precautions and considered as being potentially infectious.
- In contact with lead or copper pipes, sodium azide can generate explosive compounds.
- 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.
- Use a validated method in the laboratory to obtain a platelet neutralization by platelet factor 4, time before centrifugation should not exceed 1 hour at room temperature (18-25°C).
- When monitoring Unfractionated heparin therapy, 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.

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:
- 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 (for the United States, see the CLSI H21-A5 guidelines for further information concerning specimen collection, handling and storage).

Materials:
- Human plasma obtained from anticoagulated blood (trisodium citrate).
- Collection:
  - The blood (9 volumes) should be carefully collected onto the trisodium citrate anticoagulant (1 volume) (0.109 M) by clean venipuncture, in order to avoid any activation and platelet factor 4 release. Specific collection tubes for unfractionated heparin testing, such as the CTAD (Citrate, Thrombyline, Adenosine and Dipyridamole) tubes, can be used. The first tube must be discarded.

Centrifugation:
- When monitoring Unfractionated heparin therapy, 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 a 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:
  - 2 hours at room temperature (18-25°C).
  - 1 month at 20°C.
  - 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.

English, last revision: 08-2017
PROCEDURE:
The kit can be used for kinetic, automated or manual (endpoint) methods. Perform the test at 37°C and read color intensity at 405 nm. Whatever the method used, the assay must be performed according to the scheme reported for the manual method in order to keep a homogeneous reactivity to UFH.

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

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

<table>
<thead>
<tr>
<th></th>
<th>Microwell</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted plasma</td>
<td>12 µL</td>
<td>30 µL</td>
</tr>
<tr>
<td>Distilled water</td>
<td>24 µL</td>
<td>90 µL</td>
</tr>
<tr>
<td>R1: Substrate SXa-11 Preincubated at 37°C</td>
<td>80 µL</td>
<td>200 µL</td>
</tr>
</tbody>
</table>

Mix and incubate at 37°C, for 2-5 minutes then introduce:

<table>
<thead>
<tr>
<th></th>
<th>R2 : Factor Xa Preincubated at 37°C</th>
<th>Mix and incubate at 37°C for exactly</th>
<th>Then stop the reaction by introducing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>80 µL</td>
<td>200 µL</td>
<td>90 sec</td>
</tr>
</tbody>
</table>

Citric Acid (2%) 100 µL 500 µL Mix and measure the absorbance at 405 nm against the corresponding blank.

QUALITY CONTROL:
The BIOPHEN Heparin 3 assay can be calibrated for the assay of UFH. A specific calibrator set which covers the dynamic test range is available from HYPHEN BioMed (see the REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED paragraph) and can be used to establish the calibration curve.

Using a semi-logarithmic scale:
- The test is linear up to 1.0 IU/mL Anti-Xa for UFH.

The calibration curve shown below, obtained on STA-R² instrument with UFH calibrator is given by way of example only. The calibration curve established for the assay series must be used.

![Calibration Curve](image)

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, with the OD 405 nm along the Y-axis and the UFH concentrations expressed as IU/mL along the X-axis.
- The concentration of Heparin in the test specimen is directly inferred from the calibration curve.
- Results are expressed in anti-Xa International Units/mL (IU/mL) by reference to the International Standard (NIBSC).
- The results should be interpreted according to the patient’s clinical and biological condition.

LIMITATIONS:
- To ensure optimum test performance and to meet the specifications, the technical instructions validated by HYPHEN 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.
- Blood activation, during specimen collection and plasma preparation, may release platelet factor 4, which can inhibit heparin.
- No significant interference is observed for bilirubin concentrations <0.1 mg/mL, haemoglobin concentrations <2 mg/dL and triglycerides concentrations <1.25 mg/dL. High levels of haemoglobin or of triglycerides may affect the results. In order to get the full assay performances, the working instructions must be carefully observed.
- If the AT concentration in the tested plasma is <50%, heparin can be underestimated as the result of lack of AT. A variant protocol, with an exogenous source of AT, must then be used.
- High AT concentrations (> 150%) could interfere with the assay and mimic presence of low UFH.
- Underestimation of heparin concentration and heparin resistance has been reported in some patients with amyloidosis.

EXPECTED VALUES:
For obtaining the right efficacy along with the lowest bleeding risk, heparin dosage must be within the therapeutic range recommended by each drug manufacturer, and for each specific indication.

PERFORMANCE:
The enzymatic reaction is rapid, and allows obtaining a high sensitivity for this heparin assay.

- The detection threshold is of 0.05 IU/mL.
- Example of reproducibility obtained with plasma supplemented within UFH, when using ACL 7000 Instrument (IL)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra-assay CV (%)</th>
<th>Inter-assay CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>UFH level 1 (0.38 IU/mL)</td>
<td>2.1</td>
<td>0.93; 0.90; 0.97</td>
</tr>
<tr>
<td>UFH level 2 (0.74 IU/mL)</td>
<td>1.0</td>
<td>0.92; 0.90; 0.97</td>
</tr>
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- Correlations: The BIOPHEN Heparin assay shows good correlation with Coasmatic® Heparin performed on BCS and STA instruments, and with Rotachrom Heparin performed on STA-R² instrument (range 0-2 IU/mL):
  - Coasmatic® Heparin versus BIOPHEN Heparin (on BCS):
    - R² = 0.87; r = 0.97
  - Coasmatic® Heparin (on BCS) versus BIOPHEN Heparin (on STA-R²):
    - R² = 0.91; r = 0.99
  - BIOPHEN Heparin versus Rotachrom Heparin (on STA-R²):
    - R² = 0.97; r = 0.97

All the studies were conducted outside the US. Another study compared BIOPHEN Heparin (UFH) with Rotachrom Heparin in US:

- N = 45; y = 0.93 ± 0.007; r = 0.976

REFERENCES: