**BIOPHEN™ Heparin LRT**

**INTENDED USE:**
The BIOPHEN™ Heparin LRT kit is an anti-Xa chromogenic method for the in vitro quantitative determination of heparin and their analogs, in human citrated plasma, using a manual or automated method. This method is appropriate for the Apixaban, Rivaroxaban and Edoxaban assay, direct Factor Xa (FXa) inhibitors. This method is also appropriate for the determination of anti-Xa activity assay of Arixtra® (Fondaparinux) and Orgaran® (Sodium Danaparoid), indirect inhibitors whose activity is mediated by plasma antithrombin (AT). Reagents are in the liquid presentation, ready to use (LRT, Liquid reagent Technology).

**SUMMARY AND EXPLANATION:**

**Technical:**
Heparin is a sulfated polysaccharide with an high affinity for antithrombin (AT). When complexed with heparin, AT exhibits a fast acting and potent inhibitory activity for coagulant serine esterases: Xa, Xa, Xa, Xa and thrombin. Low Molecular Weight Heparin (LMWH), and heparin analogues, such as sodium danaparoid, inhibit more efficiently FXa than thrombin. Anti-Xa assays are then the methods of choice for measuring heparins and their analogues.1,2

The BIOPHEN™ Heparin LRT is a chromogenic anti-Xa method developed for measuring homogeneously unfractionated heparin (UFH) and LMWH, using the same calibrators curve. This method is also useful for the determination of anti-Xa activity of Orgaran® (sodium danaparoid) and Arixtra® (Fondaparinux), indirect inhibitors whose activity is mediated by plasma AT, and for the determination of direct anti-Xa inhibitors (Rivaroxaban, Apixaban and Edoxaban), using specific calibrators.

**Clinical:**
Heparin anticoagulants (UFH and LMWH) are currently used for curative or preventive indications. Alternative anticoagulant therapy (Orgaran® and Arixtra®) can be used in specific cases. Measuring these drugs concentration in patients' plasma allows monitoring the therapy and adjusting drug dosage.

Rivaroxaban, Apixaban and Edoxaban are direct oral anticoagulants (DOACs) used for monitoring the therapy and adjusting drug dosage. Measuring these drugs concentration in patients' plasma allows for the determination of direct FXa inhibitors assays.

**PRINCIPLE:**
The BIOPHEN™ Heparin LRT method is a one stage chromogenic assay based on the inhibition of a constant amount and in excess of FXa, by heparin (or other anti-Xa substance) to be assayed, in the presence of endogenous AT. The residual FXa hydrolyses a specific chromogenic substrate (Sxa-11) releasing paranitroaniline (pNA). The amount of pNA released (measured by absorbance at 405 nm) is inversely proportional to the concentration of heparin (or other anti-Xa substance) present in the reaction medium.

**Heparin and analogues:**
Heparin + AT ➔ [AT-Hep.] ➔ [FXa] ➔ [FXa-AT-Hep.] ➔ [residual FXa] ➔ [residual FXa] ➔ [Sxa-11] ➔ Peptide + pNA

Rivaroxaban / Apixaban / Edoxaban (DiXa) ➔ [DiXa] ➔ [DiXa-6] ➔ [DiXa residual] ➔ [Sxa-11] ➔ [Sxa residual] ➔ [Sxa] ➔ Peptide + pNA

**REAGENTS:**

**R1:** Reagent 1: Chromogenic substrate specific for Factor Xa (Sxa-11), liquid form.

**R2:** Reagent 2: Bovine Factor Xa, liquid form. Contains BGA, Dextran Sulfate and small amounts of sodium azide (0.9 g/L).

**Calibrators:**
- **UFH**
- **LMWH**
- **Orgaran®**
- **Arixtra®**

**Controls:**
- **Rivaroxaban standard / low range**
- **Apixaban standard / low range**
- **Edoxaban standard / low range**

**Specimen Collection:**
Clean venipuncture.

**Preparation of the specimens:**
- **Blood:** Use citrated blood (3.8% trisodium citrate, 9:1).
- **Plasma:** Use citrated plasma.
- **Serum:** Use citrated serum.

**Processing:**
- **Blood and Plasma:** Centrifugation within 30 minutes, in 15 minutes at room temperature (18°C).
- **Serum:** Centrifugation within 30 minutes, in 15 minutes at room temperature (18°C).

**STORAGE AND STABILITY:**
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.

**Reagent stability after opening:**
- 6 months at 2-8°C.
- 14 days at room temperature (18-25°C).
- Do not freeze.
- Stability on board of the analyzer: see the specific application.

**REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED:**
- Distilled water.
- 20% acetic acid or 2% citric acid (endpoint method).
- Physiological Saline (0.9% NaCl).
- Optional: Tris-NaCl-Epida buffer, pH 7.85 (AR032A/K), special dilution buffer reducing heparin interferences in direct FXa inhibitors assays.
- Specific calibrators and controls with known titration, such as:
  - **Rivaroxaban Low range**
  - **Apixaban Low range**
  - **Edoxaban Low range**

**SPECIMEN COLLECTION AND PREPARATION:**
The blood (9 volumes) should be carefully collected onto the trisodium citrate anticoagulant (1 volume) (0.109 M, 3.2%) 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.

Specimens should be prepared and stored in accordance with applicable local guidelines (for the United States, see the CLSI H21-A5® guideline for further information concerning specimen collection, handling and storage).

**ASSAY METHOD (Manual):**

1. Reconstitute the calibrators and controls as indicated in the specific instructions.
2. Dilute the specimens, calibrators (to prepare the calibration curve) and controls in physiological saline buffer, as described in the table below:

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

For an automated method, application guides are available on request. See specific application guide and specific precautions for each analyzer.

**ASSAY METHOD (Automated):**

1. Establish the calibration curve and test it with the quality controls quickly for optimal assay performance. If stored at room temperature (18-25°C), test the diluted specimens quickly. The exact calibrators and controls concentrations for each batch are indicated on the flyer provided with the kit.

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**For manual method, allow to stabilize for 30 minutes at room temperature (18-25°C), homogenize before use.**

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**For automated method, allow to stabilize for 30 minutes at room temperature (18-25°C), homogenize before use.**
3. Dispense the following to a microplate or plastic tube incubated at 37°C:

<table>
<thead>
<tr>
<th>Heparin</th>
<th>Orgaran®</th>
<th>Arixtra®</th>
<th>Rivaroxaban</th>
<th>Apixaban</th>
<th>Edoxaban</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microplate</td>
<td>Volume</td>
<td>Volume</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma to test, control or calibrator diluted</td>
<td>30 µL</td>
<td>100 µL</td>
<td>100 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RS Substrate SXa 11 Preincubated at 37°C</td>
<td>250 µL</td>
<td>250 µL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS and incubate at 37°C for 2 hours, then introduce:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2</td>
<td>Factor Xa Preincubated at 37°C</td>
<td>15 µL</td>
<td>15 µL</td>
<td>15 µL</td>
<td></td>
</tr>
<tr>
<td>MS and incubate at 37°C for exactly 60 sec.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stop the reaction by introducing:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citrate acid (3%)</td>
<td>100 µL</td>
<td>360 µL</td>
<td>400 µL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mix and measure the absorbance at 405 nm against the corresponding blank.

*a* of acetic acid (20%). The yellow color is stable for 2 hours.

The specimen blank is obtained by mixing the reagents in the reverse order to that of the test: Citric acid (3%), R2, R1, diluted specimen.

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

Create a plasma blank if sample is icoic, lipemic, haemolsed, or if its color differs from the standard plasma.

The assay of Rivaroxaban, Apixaban and Edoxaban (standard range) can be achieved by kinetics method by recording the change in absorbance between 10 and 35 seconds after the addition of FXa (i.e., ΔOD 405nm). In this case it is not necessary to subtract the blank sample, or to stop the reaction.

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.

### CALIBRATION

The BIOPHEN™ Heparin LRT assay can be calibrated for the assay of various anti-Xa analytes: Heparins and analogs, Rivaroxaban, Apixaban, Edoxaban. Kits containing plasma calibrators specific to these analytes and covering the calibration range are available from HYPHEN BioMed (see the REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED paragraph) and can be used to establish the calibration curve specific to the assayed analyte.

**Heparin, Arixtra® and Orgaran®**: The calibration curves shown below are given by way of example only. The calibration curve established for the assay series must be used.

**Apixaban, Rivaroxaban, Edoxaban**: The calibration curves shown below are given by way of example only. The calibration curve established for the assay series must be used.

### 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 concentration along the X-axis.
  - Rivaroxaban low range, Edoxaban standard range, Arixtra®, Orgaran®, UHF and LMWH, use a Lin-Log scale (concentration-OĐ).
  - Rivaroxaban standard range, Apixaban and Edoxaban Low range, use a Log-Log scale (concentration-OĐ).

When employing the kinetic method, use ΔOD 405 instead of OD 405.

- The concentration of heparin (or other anti-Xa molecule) in the test specimen is directly inferred from the calibration curve, if the standard dilution is used.

- If other dilutions are used, the level obtained should be multiplied by the additional dilution factor used.
- Results are expressed in International Units/mL (IU/mL) for Heparin, in U/mL for Orgaran®, in µg/mL for Arixtra®, or in ng/mL for Rivaroxaban, Apixaban and Edoxaban.

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.
- 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 samples due to collection and plasma preparation, may induce release of Platelet Factor 4 (PF4). PF4 is an inhibitor of heparin. This assay was designed for minimizing the interference of anti-heparin substances in plasma, and especially that of PF4.
- If the AT concentration in the tested plasma is <50%, heparin, Arixtra® or Orgaran® can be underestimated as the result of lack of factor Xa AT must be confirmed by an assay. A variant protocol, with an exogenous source of AT, must then be used. High AT concentrations (>150%) could interfere with the assay.
- High concentration sample may be diluted in a normal plasma pool.
- Underestimation of heparin concentration and heparin resistance has been reported in some patients with amyloidosis.
- When a unique curve is used (LMWH/UFH), check that the instrument and application allow a good superimposition between LMWH and UFH calibrations.

### EXPECTED VALUES

Anti-Xa drugs are absent from normal plasma. For obtaining the right efficacy along with the lowest bleeding risk, the heparin dosage must be within the therapeutic range recommended by each drug manufacturer, and for each specific indication

Normal range, therapeutic range and bleeding risk range (according to the drug) should be defined according to the current local recommendations.

### PERFORMANCES

- The lower limit and the measurement range are defined by the analytical system used.
- For the standard range, the calibration range is of about 0 to 600 ng/mL Rivaroxaban/Edoxaban/Apixaban.
- For the low range, the calibration range is of about 0 to 100 ng/mL Rivaroxaban and of about 0 to 120 ng/mL Apixaban/Edoxaban.

- The calibration range is of about 0 to 1.50 U/mL for UHF, 0 to 1.75 U/mL for LMWH, 0.10 to 1.60 µg/mL for Arixtra® and 0 to 1.75 U/mL for Orgaran®.
- The enzymatic reaction is rapid, and allows obtaining a high sensitivity for this assay.
- Performance studies were conducted internally using CS-series. Performance was assessed using laboratory controls over a minimum of 10 series and at least one repetition for each control level. The following results were obtained:

#### Intra assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>UHF level</th>
<th>LMWH level</th>
<th>Orgaran level</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.19 IU/mL</td>
<td>0.98 IU/mL</td>
<td>1.00 IU/mL</td>
</tr>
<tr>
<td>10</td>
<td>0.88 IU/mL</td>
<td>0.98 IU/mL</td>
<td>1.00 IU/mL</td>
</tr>
<tr>
<td>5</td>
<td>0.56 IU/mL</td>
<td>0.98 IU/mL</td>
<td>1.00 IU/mL</td>
</tr>
</tbody>
</table>

#### Inter assay

<table>
<thead>
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</tbody>
</table>

- Correlation with reference methods (ICMS M S versus BIOPHEN™ Heparin LRT, Epoxyland):

Systex CS-5100: r = 0.998, y = 0.98x + 0.23

For other molecules, refer to the specific application guide of the analyzer used.

- Interferences: see specific application guide for each analyzer.

### REFERENCES

10. CBH Document H21 All “Collection, transport, and processing of blood specimens for testing of plasma based coagulation assays and molecular heparin assays; approved guideline”. (2008).

### SYMBOLS

- Symbols used and signs listed in the ISO 15223-1 standard, see Symbol definitions document.
- HELA 117: May cause an allergic skin reaction.
- Changes compared to the previous version.