BIOPHEN™ Heparin LRT

REF 221015-RUO

R1 R2 4 x 5 mL

Anti-Xa chromogenic method for Heparin and their analogs assay, with liquid reagents ready to use.

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

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INTENDED USE:

The BIOPHEN™ Heparin LRT kit is a chromogenic method proposed for the quantitative determination of heparin and theirs analogs, in human citrated plasma using anti-Xa manual or automated method. This method is appropriate for the Apixaban, Rivaroxaban and Edoxaban assay, direct Factor Xa inhibitor. This method is also appropriate for the determination of anti-Xa activity assay of Arixtra® (Fondaparinux) and Orgaran® (Sodium Danaparoïd), indirect inhibitor whose activity is mediated by AT plasma. The reagents are in liquid form ready to use.
This kit should be used for research use only and must not be used for patient diagnosis or treatment.

SUMMARY AND EXPLANATION:

The BIOPHEN™ Heparin LRT kit is a chromogenic anti-Xa method. Heparin is a natural sulphated polysaccharide with a high affinity for antithrombin (AT). Complexed with heparin, antithrombin exhibits a fast acting and potent inhibitory activity for coagulant serine esterases: Factors IXa, Xa, XIIa, XIa and thrombin[1,2].
This method is developed for measuring homogeneously heparin (UFH) and Low Molecular Weight Heparin (LMWH) using the same calibration curve, provided that the application used allows this superimposition.
Determination of other anti-Xa inhibitors (Rivaroxaban, Apixaban, Edoxaban, Arixtra® and Orgaran®) is performed using specific calibration of the molecule to be assayed.

PRINCIPLE:

The BIOPHEN™ Heparin LRT method is an assay based on the inhibition of a constant and in excess amount of Factor Xa, by heparin (or other anti-Xa substance) to be assayed, in the presence of endogenous antithrombin. The residual Factor Xa hydrolyses a specific chromogenic substrate (SXa-11) releasing paranitroaniline (pNA)[3]. The quantity of released pNA (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 theirs analogues
Heparin + AT ➔ [AT Hep.]
[AT Hep.] + [FXa (excess)] ➔ [FXa-AT-Hep.] + [residual FXa]
[FXa (residual)] + SXa-11 ➔ Peptide + pNA
Rivaroxaban / Apixaban / Edoxaban (DiXaI)
[DiXaI] + [FXa (excess)] ➔ [FXa-DiXaI] + [FXa residual]
[FXa (residual)] + Substrate ➔ Peptide + pNA

REAGENTS:

R1 Reagent 1: Chromogenic substrate specific for Factor Xa (SXa-11), liquid form.
4 vials of 5 mL.
R2 Reagent 2: Bovine Factor Xa, liquid form. Contains BSA.
4 vials of 5 mL.

Reagent R2 contains Dextran Sulfate[4] and small amounts of sodium azide (0.9 g/L), 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.
- 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's stability in the analyzer.
- 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 shipped at room temperature over a short period of time, without degradation.
- This material contains substances of animal origin and must be handled as a carrier and a potential transmitter of diseases.
- Create a plasma blank if this latter is icteric, lipaemic, haemolysed, or if its color differs from the standard plasmas.
- When employing the kinetic method, use ΔOD 405 instead of OD 405.
- For *in vitro* use.

R1 H317 : May cause an allergic skin reaction.

REAGENT PREPARATION AND STABILITY:

R1 **Reagent 1**: **Factor Xa specific chromogenic substrate (SXa-11)**
Brown vial. Ready to use. Allow to stabilize for 30 minutes at room temperature (18-25°C), before use. Homogenize the reagent prior to use.
Reagent stability after opening, excluding any contamination or evaporation, and stored in the original vial, is of:
- **6 months** at 2-8°C.
- **14 days** at room temperature (18-25°C).
- **Do not freeze.**

R2 **Reagent 2**: **Factor Xa**
Clear vial. Ready to use. Allow to stabilize for 30 minutes at room temperature (18-25°C), before use. Homogenize the reagent prior to use.
Reagent stability after opening, excluding any contamination or evaporation, and stored in the original vial, is of:
- **6 months** at 2-8°C.
- **14 days** at room temperature (18-25°C).
- **Do not freeze.**

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 (end point method).
- Physiological Saline (0.9% NaCl).
- Specific calibrators and controls with known titration, such as:

	UFH	LMWH	Orgaran®	Arixtra®
Calibrators	222301-RUO	222001-RUO	222201-RUO	222501-RUO
Controls	223101-RUO / 224101-RUO / 223901-RUO	223001-RUO / 223801-RUO / 224201-RUO / 223701-RUO / 224301-RUO / 224401-RUO	223501-RUO	224001-RUO

	Rivaroxaban standard / low range	Apixaban standard / low range	Edoxaban standard / low range
Calibrators	222701-RUO / 226001-RUO	226201-RUO / 226101-RUO	226501-RUO / 226401-RUO
Controls	224501-RUO / 225101-RUO	225301-RUO / 225201-RUO	225501-RUO / 225401-RUO

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 (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, Theophylline, Adenosine and Dipyridamole) tubes, can be used. The first tube must be discarded.
- 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 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:

For Direct Oral Anti-Coagulants[5] :
  - 4 hours at room temperature (18-25°C).
  - 2 weeks at -20°C.
  - 24 months at -70°C.

For Heparin and Orgaran® :
  - 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.

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**.

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

Assay of Heparin, Orgaran® or Arixtra® (Manual method):
1. Reconstitute the calibrators and controls as indicated in the specific inserts and diluted at dilution described in the table below. Run the calibration curve and test it with quality controls within one hour for optimal assay performance
The samples, controls and calibrators should be diluted as described in the table below:

Products	Calibrators Reference	Controls Reference	Dilution in physiological saline
LMWH	222001-RUO	223001-RUO / 223801-RUO / 224201-RUO / 223701-RUO / 224301-RUO / 224401-RUO	**1:2**
UFH	222301-RUO	223101-RUO / 224101-RUO / 223901-RUO	**1:2**
Arixtra®	222501-RUO	224001-RUO	**1:2**
Orgaran®	222201-RUO	223501-RUO	**1:2**

Dilution should be tested in the hour. Please note that the exact concentration of the calibrators and controls is indicated for each lot on the flyer provided with the kit.

2. Dispense the following to a plastic tube incubated at **37°C**:

	Microplate	Volume
Plasma to test, control or calibrator diluted	30 µL	100 µL
R1 Substrate SXa-11 Preincubated at **37°C**	75 µL	250 µL
Mix and incubate at **37°C**, for **2 minutes**, then introduce:		
R2 Factor Xa Preincubated at **37°C**	75 µL	250 µL
Mix and incubate at **37°C** for **exactly:**	60 sec.	60 sec.
Stop the reaction by introducing:		
Citric acid (2%)*	100 µL	350 µL
Mix and measure the absorbance at **405nm** against the corresponding blank.		

*Or Acid Acetic (20%). The yellow color is stable for 2 hours.
The sample blank is obtained by mixing the reagents in the reverse order from that of the test i.e.: Citric acid (2%), Factor Xa, substrate, diluted plasma.
Measure the absorbance **at 405 nm**. The sample blank value must be deduced from the absorbance measured for the corresponding assay.

Assay of Rivaroxaban, Apixaban and Edoxaban (Manual method):

1. Reconstitute the calibrators and controls as indicated in the specific inserts and diluted at dilution described in the table below. Run the calibration curve and test it with quality controls within one hour for optimal assay performance

The samples, controls and calibrators should be diluted as described below in the table below:

Products	Calibrators Reference	Controls Reference	Dilution in physiological saline
Rivaroxaban Standard range	222701-RUO	224501-RUO	**1:10**
Rivaroxaban Low range	226001-RUO	225101-RUO	**1:3**
Apixaban Standard range	226201-RUO	225301-RUO	**1:15**
Apixaban Low range	226101-RUO	225201-RUO	**1:3**
Edoxaban Standard range	226501-RUO	225501-RUO	**1:10**
Edoxaban Low range	226401-RUO	225401-RUO	**1:2**

Dilution should be tested in the hour. Please note that the exact concentration of the calibrators and controls is indicated for each lot on the flyer provided with the kit.

2. Introduce, in a plastic tube incubated at 37°C:

	Volume
Plasma to test, control or calibrator diluted.	100 µL
R1 Substrate SXa-11 Preincubated at **37°C**	250 µL
Mix and incubate at **37°C**, for **2 minutes**, then introduce:	
R2 Factor Xa Preincubated **at 37°C**	250 µL
Mix and incubated at **37°C** for **exactly 120 sec.**	
Stop the reaction by introducing:	
Citric acid (2%)*	400 µL
Mix and measure the absorbance **at 405nm** against the corresponding blank	

*Or Acid Acetic (20%). The yellow color is stable for 2 hours.

The sample blank is obtained by mixing the reagents in the reverse order from that of the test i.e.: Citric acid (2%), Factor Xa, substrate, diluted plasma.

Measure the absorbance at **405 nm**. The sample blank value must be deduced from the absorbance measured for the corresponding assay.

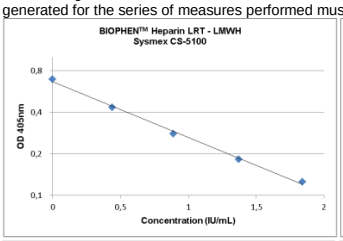
The assay of Rivaroxaban, Apixaban and Edoxaban (standard range) can be achieved by kinetics method by recording the change in absorbance between 10 and 35 seconds after the addition of Xa (i.e., OD 405 nm). In this case it is not necessary to subtract the blank sample, or to stop the reaction.

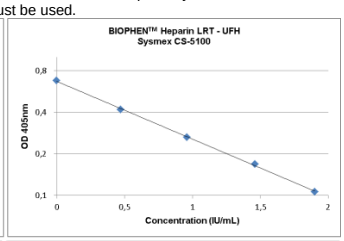
If a reaction volume other than that specified above is required for the method used, the ratio of volumes must be strictly observed to guarantee assay performance. The user is responsible for validating any changes and their impact on all results.

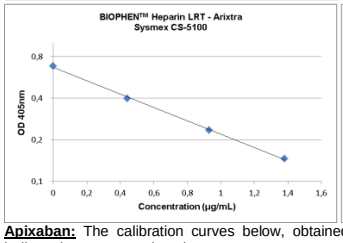
CALIBRATION:

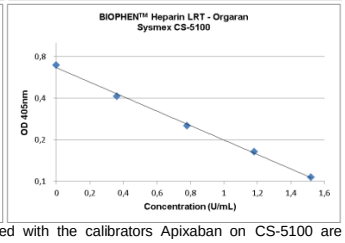
The plasma calibrators covering the dynamic test range are available from HYPHEN BioMed (see the "REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED" paragraph) and can be used to establish the calibration curve.

Heparin, Arixtra® and Orgaran®: The calibration curves below, obtained with the calibrators UFH, LMWH, Orgaran® and Arixtra® on CS-5100 are indicated as an example only. The calibration curve generated for the series of measures performed must be used.



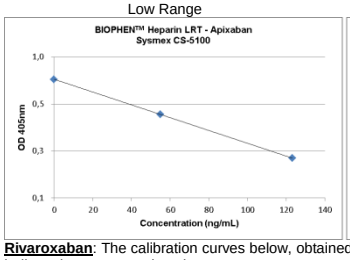




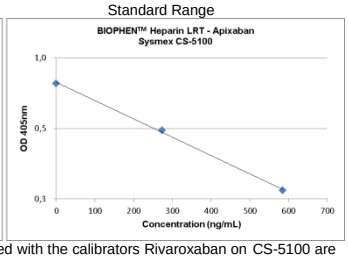


Apixaban: The calibration curves below, obtained with the calibrators Apixaban on CS-5100 are indicated as an example only.

Low Range

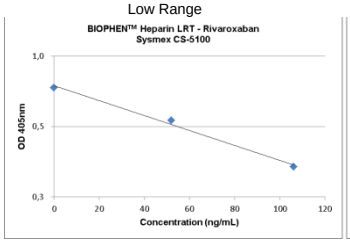


Standard Range

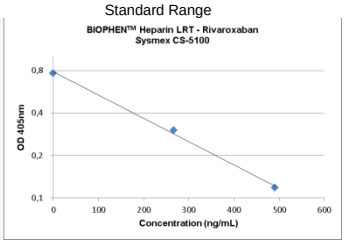


Rivaroxaban: The calibration curves below, obtained with the calibrators Rivaroxaban on CS-5100 are indicated as an example only.

Low Range

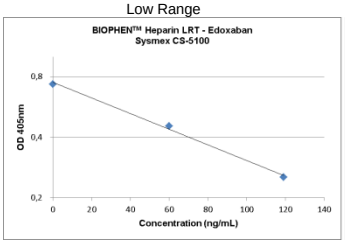


Standard Range

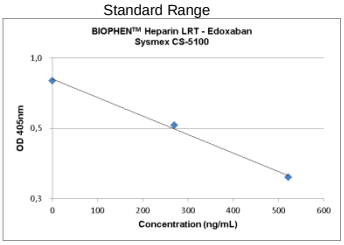


Edoxaban: The calibration curves below, obtained with the calibrators Edoxaban on CS-5100 are indicated as an example only.

Low Range



Standard Range



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® UFH and LMWH**, use a **Lin-Log** scale (ng/mL – OD).
  - **Rivaroxaban standard range, Apixaban and Edoxaban Low range,** use a **Lin-Lin** scale (ng/mL – OD).
- 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.
- Results are expressed in International Units/mL (IU/mL), in U/mL for Orgaran® , in µg/mL for Arixtra®, or in ng/mL for Rivaroxaban, Apixaban and Edoxaban.

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

LIMITATIONS:

- Blood activation during 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.
- No significant interference on heparin determination is observed for bilirubin concentrations <0.1 mg/mL, haemoglobin concentrations <2 mg/mL and triglycerides concentrations <1.25mg/mL added to plasma. High levels of haemoglobin or of triglycerides may affect the results.
- If the AT concentration in the tested plasma is <50%, heparin, Arixtra® or Orgaran® can be underestimated as the result of lack of AT (the lack of 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.
- When a unique curve is used (LMWH/UFH), check that the instrument and application used allow a good superimposition between LMWH and UFH calibrations.
- 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.
- Any plasma displaying a coagulum or showing signs of contamination must be rejected.

PERFORMANCE:

- **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 and of about 0 to 700 ng/mL 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 about **0 to 1.50 IU/mL for UFH, 0 to 1.75 IU/mL for LMWH, 0 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 heparin assay.
- Performance studies were conducted internally on 1 batch of reagent using CS series. Performance was assessed using laboratory controls over a minimum of 10 series and at least one repetition per series for each control level. The following results were obtained:

	Intra assay				Inter assays			
Sample	**n**	**Mean**	**CV%**	**SD**	**N**	**Mean**	**CV%**	**SD**
UFH level 1	10	0.19 IU/mL	2.80	0.01	20	0.20 IU/mL	5.90	0.01
UFH level 2	10	0.56 IU/mL	0.90	0.01	20	0.57 IU/mL	1.50	0.01
LMWH level 3	20	0.78 IU/mL	1.00	0.01	20	0.80 IU/mL	1.10	0.01
LMWH level 4	20	1.22 IU/mL	0.60	0.01	20	1.18 IU/mL	1.20	0.02
Rivaroxaban	30	317.3 ng/mL	0.88	2.80	20	315.8 ng/mL	1.32	4.16
Rivaroxaban Low	30	80.5 ng/mL	0.67	0.53	22	85.3 ng/mL	4.12	3.51
Apixaban	30	207 ng/mL	1.22	2.53	20	212.3 ng/mL	2.63	5.58
Apixaban Low	30	84.6 ng/mL	1.15	0.98	20	83.9 ng/mL	2.16	1.82
Edoxaban	40	296 ng/mL	1.50	4.40	120	305 ng/mL	2.30	7.00
Edoxaban Low	40	26.1 ng/mL	1.90	0.50	120	27.5 ng/mL	5.00	1.40
Orgaran®	10	1.00 U/mL	0.52	0.01	10	1.00 U/mL	0.88	0.01
Arixtra®	10	1.18 µg/mL	0.57	0.01	10	1.19 µg/mL	0.67	0.01

REFERENCES:

1. Hemker HC and Beguin S. The mode of action of heparin In-vitro and in-vivo. Adv Exp Med Biol (1992).
2. Leslie B *et al.* Investigation of the anticoagulant mechanism of a covalent antithrombin-heparin complex J Biol Chem (2000).
3. Marlowe CK *et al.* Design synthesis and structure activity relationship of a series of arginine aldehydes factor Xa Inhibitors. Part 1: structure based on the (D)-Arg-Gly-Arg tripeptide sequence. Bioorg Med Chem Lett (2000).
4. Lyon SG *et al.* Modification of an Amidolytic Heparin Assay to Express Protein-Bound Heparin and to Correct for the Effect of Antithrombin III Concentration. Thromb Hemost (1987).
5. Douxfils J, *et al.* Non-VKA Oral Anticoagulants: Accurate Measurement of Plasma Drug. BioMed Research International. 2015.

SYMBOLS:

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

Changes compared to the previous version.