BIOPHEN™ ANTI-Xa
(2 Stages Heparin Assay)

Ref 221005

(R1, R2, R3 : 2 x 1 mL)

Two stages Anti-Xa chromogenic method for the Heparin activity measurement on plasma or purified medium, according to Pharmacopeia (USP, EP).

FOR RESEARCH USE ONLY.

DO NOT USE IN DIAGNOSTIC PROCEDURES.

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INTENDED USE:

This BIOPHEN™ ANTI-Xa (2 Stages Heparin Assay) kit is a two-stage chromogenic assay for measuring the activity of heparins (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 sulphated polysaccharide with a high affinity for antithrombin. Complexed with heparin, antithrombin exhibits a fast acting and potent inhibitory activity for coagulant serine esterases: IXa, Xa and thrombin. Low Molecular Weight Heparin (LMWH), and analogues such as Sodium Danaparoïd, inhibit more efficiently Factor Xa than thrombin, whereas the Unfractionated Heparin (UFH) inhibit more efficiently thrombin than other serine esterases. The Pentasaccharide (Arixtra®) inhibits more specifically Factor Xa.
This heparin assay is a two-stage assay for measuring accurately and sensitively heparin concentrations in plasma or in purified systems. Tested plasma needs to be diluted before assaying it.
This assay, using a predilution of Antithrombin and Factor Xa reagents in specific buffer (not provided within this kit), is in compliance with the United States Pharmacopoeia (USP)[4] and European Pharmacopoeia (EP)[5].

PRINCIPLE:

The BIOPHEN™ ANTI-Xa (2 Stages Heparin Assay) kit is a chromogenic anti-Xa method, developed for measuring Unfractionated Heparins (UFH) and Low Molecular Weight Heparins (LMWH) in plasma or in purified solutions, for their Anti-Xa activity.
The BIOPHEN™ ANTI-Xa (2 Stages Heparin Assay) assay is a method based on the inhibition of a constant amount of Factor Xa (FXa), by the tested heparin in presence of exogenous antithrombin (stage 1), and hydrolysis of a Factor Xa specific chromogenic substrate (CS11(65)), by the Factor Xa 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 Factor Xa activity. There is an inverse relationship between the concentration of heparin and color development.

Heparin + AT ➔[AT Hep.]
[AT Hep.] + [FXa (excess)] ➔ [FXa-AT-Hep.] + [residual FXa]
[residual FXa] + Substrate ➔ Peptide + pNA

REAGENTS:

R1: Reagent 1 : ATIII (h)
Human Antithrombin (ATIII), lyophilized vial containing about 5 IU/mL. Contains BSA.
2 vials of 1 mL.

R2: Reagent 2 : FXa (b)
Purified bovine Factor Xa, lyophilized vial containing about 40 µg (i.e. about 90nkats, when determined in optimized conditions with CS-11(22) specific substrate). Factor Xa concentration is exactly adjusted from lot to lot for offering an optimized assay reactivity and linearity.
2 vials of 1 mL.

R3: Reagent 3 : Factor Xa specific chromogenic substrate
Chromogenic substrate specific for Factor Xa (CS-11(65)), vial of about 4 mg (about 6 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's stability in the analyzer.
- The human plasma used to prepare the human antithrombin 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 and Factor Xa has been tested by recorded methods and is certified free of infectious agents, in particular the causative agent of bovine spongiform encephalitis.
- 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.
- 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.
- The Factor Xa and AT concentrations are adjusted if required for each lot for providing the right reactivity in the assay.
- 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 restored 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.
- **7 days** at room temperature (18-25°C).
- **2 months** frozen at -20°C or less*

R2: Reagent 2: **FXa (b)**
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 restored Factor Xa).
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.
- **7 days** at room temperature (18-25°C).
- **2 months** frozen at -20°C or less*

R3: Reagent 3: **Factor Xa specific chromogenic substrate**
Reconstitute the contents of each vial with exactly **1 mL of 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** extemporaneously in the appropriate buffer (see table below, if the whole vial is used, add **4 mL** of specific buffer to the **1 mL** of restored substrate).
Homogenize the reagent prior to use.
Reagent stability after reconstitution, excluding any contamination or evaporation, and stored in the original vial, is of:

- **2 months** at 2-8°C.
- **7 days** at room temperature (18-25°C).
- **2 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 should be checked in the working conditions of the laboratory user.

	Dilution of reagent		Volume of buffer (for 1 mL of reagent)		Buffer used	
Heparin measured	**LMWH**	**UFH**	**LMWH**	**UFH**	**LMWH**	**UFH**
R1	1/5	1/5	4mL	4mL	AR005L	AR030K
R2	1/5	1/5	4mL	4mL	AR005L	AR030K
R3	1/5	1/5	4mL	4mL	AR029K	Distilled water

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).
- Specific buffers such as:

Product Name	Reference
Tris-EDTA-NaCl-PEG, pH 8.40	AR030K
Tris-NaCl-BSA, pH 7.40	AR005L
Tris-NaCl, pH 7.40	AR028K
Tris-EDTA-NaCl, pH 8.40	AR029K

- Calibrators and controls with known titration for Heparin to be assayed.
- For plasma assay, it is possible to use following calibrators and controls:

Product Name	Reference
BIOPHEN UFH Control Plasma	223101-RUO
BIOPHEN LMWH Control Plasma	223001-RUO
BIOPHEN LMWH Control Low	223701-RUO
BIOPHEN Heparin Calibrator	222001-RUO
BIOPHEN UFH Calibrator	222301-RUO

- International reference, compliant with pharmacopoeia used or internal reference material, specific for heparin to measure.

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. Specific collection tubes for unfractionated heparin testing, such as the CTAD (Citrate, Theophylline, 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[6, 7]:
  - 4 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 **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 samples) 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:

Concentration LMWH (IU/mL)	0.09	0.25	0.50	0.75	1.00
LMWH solution at 1IU/mL	90µL	250µL	500µL	750µL	1mL
Specific buffer	910µL	750µL	500µL	250µL	-

Concentration UFH (IU/mL)	0.09	0.25	0.50	0.75	1.00
UFH solution at 1IU/mL	90µL	250µL	500µL	750µL	1mL
Specific buffer	910µL	750µL	500µL	250µL	-

	Dilution		Specific buffer	
	LMWH	UFH	LMWH	UFH
Calibrators	1/15	1/15	AR005L (EP) or AR028K (USP)	AR030K

For the plasma, it is possible to use calibrators available (ie. BIOPHEN Heparin Calibrator 222001-RUO or 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:

Sample	Reference	Dilution	Specific buffer
BIOPHEN LMWH Control Low	223701-RUO	**1/15**	**AR005L (EP) or AR028K (USP)**
BIOPHEN UFH Control plasma	223101-RUO	**1/15**	**AR030K**
LMWH samples	n.a.	**1/15**	**AR005L (EP) or AR028K (USP)**
UFH samples	n.a.	**1/15**	**AR030K**

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**:

	Microplate	Volume
Sample, calibrator or control diluted	40 µL	200 µL
R1: Antithrombin Preincubated at **37°C**	40 µL	200 µL
Mix and incubate at **37°C**, for **2 minutes**, then introduce:		
R2: Factor Xa Preincubated at **37°C**	40 µL	200 µL
Mix and incubate at **37°C**, for exactly **2 minutes**, then introduce:		
R3: Substrate Preincubated at **37°C**	40 µL	200 µL
Mix and incubate at **37°C** for **exactly:**	2 min	90 sec
Stop the reaction by introducing:		
Citric acid (2%)*	80 µL	400 µL
Mix and measure the absorbance at **405 nm** against the corresponding blank.		

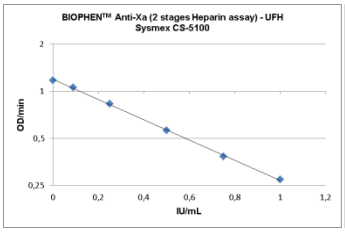
*Or acetic acid (20%). The yellow color is stable for 2 hours.
The sample blank is obtained by mixing the reagents in the reverse order to that of the test: Citric acid (2%), R3, R2, R1, dilute sample.
Measure the optical density at 405 nm. Subtract the measured blank value from the absorbance measured for the corresponding test.

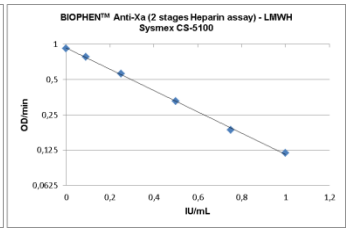
If a reaction volume other than that specified above is required for the method used, the ratio of volumes must be strictly observed to guarantee assay performance. The user is responsible for validating any changes and their impact on all results.

CALIBRATION:

The BIOPHEN™ ANTI-Xa (2 Stages Heparin Assay) assay can be calibrated for the assay of LMWH, UFH and their analogs. Calibrators and controls specific kit covering 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.

The calibration curves on CS-series 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 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. x15 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 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.
- If a higher working range for heparin is required, the standard assay dilution (d=1:15) can be adjusted accordingly. For example, use a 1:30 dilution (i.e. d:2) for a working range from 0 to 2 IU/mL in plasma. The heparin concentrations measured must then be multiplied by the dilution factor used.
- Lin-Log calibration mode used as per Pharmacopoeia, but Lin-Lin calibration mode may improve linearity for UFH.
- 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 according to USP. At very high dilution, the addition of the carrier molecule (BSA type) is likely to improve the robustness of the results.

REFERENCES:

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6. Woodhams B. *et al*. Stability of coagulation proteins in frozen plasma. Blood coagulation and Fibrinolysis. 2001.
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SYMBOLS:

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

Changes compared to the previous version.