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 Pharmacopeia (USP, EP).

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

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INTENDED USE:

This BIOPHEN™ ANTI-IIa (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:

Technical:
Heparin is a sulphated polysaccharide with a high affinity for antithrombin. When complexed with heparin, antithrombin exhibits a fast acting and potent inhibitory activity for coagulant serine esterases: IXa, Xa and thrombin. The Low Molecular Weight Heparin (LMWH) and heparin analogues, such as Sodium Danaparoïd, inhibit more efficiently Factor Xa than thrombin, whilst Unfractionated Heparin (UFH) inhibits efficiently thrombin and also the other serine esterases. Anti-IIa assays are then the right methods for measuring the anti-thrombin activity of large heparin molecules[1].
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 needs to be diluted before assaying it.
Purified human thrombin used in the assay is mainly present in the α form (obtained by direct activation of Prothrombin) which, for an equivalent concentration in chromogenic activity, exhibits a higher coagulant reactivity than "degraded" β or γ forms.
This assay, using a predilution of Antithrombin and Thrombin reagents in specific buffer (not provided within this kit), is in compliance with the United States Pharmacopoeia (USP)[2] and European Pharmacopoeia (EP)[3].

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-01(38)), 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.

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

REAGENTS:

R1 **ATIII (h) :** Human Antithrombin (ATIII), lyophilized vial containing about 1.25 IU/mL. Contains BSA.
R2 **Purified human Thrombin**, mainly in the α form, lyophilized vial containing about 120 NIH (or IU), or about 150 nkats (when determined in optimized conditions with CS-01(38) specific substrate).
R3 **Chromogenic substrate specific for Thrombin (CS-01(38)),** vial of about 6.25 mg, lyophilized in presence of mannitol.

R1 R2 R3 **2 vials of 1 mL.**

WARNINGS AND PRECAUTIONS:

- Some reagents provided in these kits contain materials of human and animal origin. Whenever human plasma is required for the preparation of these reagents, approved methods are used to test the plasma for the antibodies to HIV 1, HIV 2 and HCV, and for hepatitis B surface antigen, and results are found to be negative. However, no test method can offer complete assurance that infectious agents are absent. Therefore, users of reagents of these types must exercise extreme care in full compliance with safety precautions in the manipulation of these biological materials as if they were infectious.
- Waste should be disposed of in accordance with applicable local regulations.
- Use only the reagents from the same batch of kits.
- Aging studies show that the reagents can be shipped at room temperature without degradation.
- 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.
- This device of *in vitro* use is intended for professional use in the laboratory.

REAGENT PREPARATION:

Gently remove the freeze-drying stopper, to avoid any product loss when opening the vial.

R1 R2 Reconstitute the contents of each vial with exactly **1 mL of distilled water.**
Shake vigorously until complete dissolution while avoiding formation of foam, allow to stabilize for 30 minutes at room temperature (18-25°C) and homogenize. Just before use, dilute **1/5** in the appropriate buffer according to the Heparin to be assayed (see table below or application guide instructions, if the whole vial is used, add **4 mL** of buffer to the restored **1 mL**).

R3 Reconstitute the contents of each vial with exactly **1 mL of distilled water.**
Shake vigorously until complete dissolution while avoiding formation of foam, allow to stabilize for 30 minutes at room temperature (18-25°C) and homogenize. Just before use, dilute **1/5** (extemporaneously for manual method) in the appropriate buffer depending on the heparin to be assayed (see table below or application guide instructions, if the whole vial is used, add **4 mL** of specific buffer to the **1 mL** of restored substrate).

	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 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.

R1 R2 R3 Reagent stability after reconstitution, free from any contamination or evaporation, and stored closed, is of:

- **15 days** at 2-8°C.
- **4 days** at room temperature (18-25°C).
- **6 months** frozen at -20°C or less*
- **Stability on board of the analyzer: see the specific application.**

*Thaw only once, as rapidly as possible at 37°C and use immediately.

Stability of diluted reagents should be checked in the working conditions of the laboratory user.

If the substrate become yellow, this indicate a contamination. Discard the vial and use a new one.

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 calibrator and control:

Product Name	Reference
BIOPHEN™ UFH Control Plasma*	223101-RUO
BIOPHEN™ UFH Calibrator*	222301-RUO

* plasmas titrated in anti-Xa activity.

- International reference, compliant with the 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:

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, Theophylline, 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[4] guideline for further information concerning specimen collection, handling and storage).
For plasma storage, please refer to references[5,6].

PROCEDURE:

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

For an automated method, application guides are available on request. See specific application guide 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:

Concentration LMWH (IU/mL)	0.135	0.25	0.50	0.75	1.00
LMWH solution at 1IU/mL	135µL	250µL	500µL	750µL	1mL
Specific buffer	865µL	750µL	500µL	250µL	-

Concentration UFH (IU/mL)	0.135	0.25	0.50	0.75	1.00
UFH solution at 1IU/mL	135µL	250µL	500µL	750µL	1mL
Specific buffer	865µL	750µL	500µL	250µL	-

	Dilution		Specific Buffer	
	LMWH	UFH	LMWH	UFH
Calibrators	1:25	1:25	AR005L (EP) or AR028K (USP)	AR030K

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:

Sample	Reference	Dilution	Buffer
LMWH Control plasma	n.a.	**1/25**	**AR005L (EP) or AR028K (USP)**
BIOPHEN™ UFH Control plasma	223101-RUO	**1/25**	**AR030K**
LMWH samples	n.a.	**1/25**	**AR005L (EP) or AR028K (USP)**
UFH samples	n.a.	**1/25**	**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	Tube
Sample, calibrator or control diluted	40 µL	200 µL
R1 Human Antithrombin Preincubated at **37°C**	40 µL	200 µL
Mix and incubate at **37°C**, for **2 minutes**, then introduce:		
R2 Human Thrombin 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.

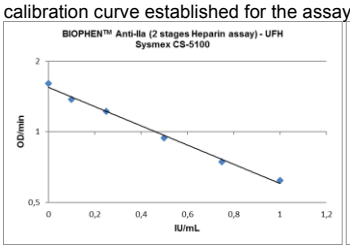
Measure the optical density at **405 nm**. Subtract the measured blank value from the absorbance measured for the corresponding test.
Create a plasma blank if sample is icteric, lipaemic, haemolysed, or if its color differs from the standard plasmas.

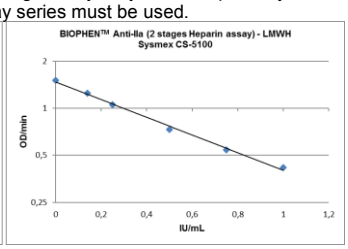
If a reaction volume other than that specified above is required for the method used, the ratio of volumes must be strictly observed to guarantee assay performance. The user is responsible for validating any changes and their impact on all results.

CALIBRATION:

The BIOPHEN™ ANTI-IIa (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 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 established, 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 acceptance range for the method.
Each laboratory must define its acceptance ranges and verify the expected performance in its analytical system.

RESULTS:

- For the manual endpoint method, plot the calibration curve log-lin, with the OD 405 nm (log) along the Y-axis and the Heparin concentration, expressed as IU/mL, along the X-axis.
- When employing the kinetic method, use ΔOD 405 instead of OD 405.
- The concentration of Heparin (IU/mL) in the test specimen is directly inferred from the calibration curve, when the standard dilution is used.
- If other dilutions are used, the level obtained should be multiplied by the additional dilution factor used.

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.
- 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.
- 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 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:

1. Van Putten J et al. Automated spectrophotometric heparin assays. Comparison of methods. Haemostasis, 14, 195-204, 1984.
2. USP40, effective May 1, 2017.
3. Ph. Eur., 9th Edition, effective January 1, 2017.
4. CLSI Document H21-A5: "Collection, transport, and processing of blood specimens for testing plasma -based coagulation assays and molecular hemostasis assays; approved guideline". 2008
5. Mauge L. and Alhenc-Gelas M. Stabilité pré-analytique des paramètres de la coagulation: revue des données disponibles. Ann Biol Clin. 2014.
6. Woodhams B. et al. Stability of coagulation proteins in frozen plasma. Blood coagulation and Fibrinolysis. 2001.

SYMBOLS:

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

Changes compared to the previous version.