

**BIOPHEN**  
**HEPARIN ANTI-IIa (2 stages)**

REF: 221025

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

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**INTENDED USE:**

This Heparin Anti-IIa method is a two stage chromogenic assay for measuring the concentration of heparin, and heparin like anticoagulants, using an automatic or a manual method, for heparin concentration ranges from 0.002 to 0.04 IU/ml (\*) in the tested dilution (or from 0 to 1 IU/ml in plasma tested diluted 1:25). This method can be used for testing heparin in human citrated plasma, or in purified solutions.

**This kit is for research use only and should not be used for patient diagnosis or treatment.**

**TEST PRINCIPLE:**

The Heparin Anti-IIa method is a two stage chromogenic anti-IIa assay developed for measuring heparin (UFH), or heparin like anticoagulants, in human citrated plasma, or in purified solutions, for their Anti-IIa activity.

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. LMWH, and heparin analogues, such as Sodium Danaparoid, inhibit more efficiently Factor Xa than thrombin, whilst 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.

The Heparin Anti-IIa 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, then hydrolysis of a Thrombin specific chromogenic substrate, by Thrombin in excess. pNA is then released from the substrate. The amount of pNA released is then a relation of the residual Thrombin activity. There is an inverse relationship between the concentration of heparin and color development, measured at 405 nm.

Heparin + AT → [AT Hep.]

[AT Hep.] + [IIa (excess)] → [FIIa-AT-Hep.] + [residual FIIa]  
[FIIa (residual)] + IIa-Subs. → Peptide + pNA

**REAGENTS SUPPLIED:**

The Heparin Anti-IIa (Plasma) kit contains 2 vials of human Antithrombin (AT), 2 vials of human Thrombin, 2 vials of a specific Thrombin substrate and 2 vials of assay reaction buffer.

**Reagent 1 (R1): ATIII (h):**

Human Antithrombin (AT), lyophilized vial containing about 1.25 IU:  
2 vials (each vial to be restored with 1 mL of distilled water, then diluted 1:5 in R4 buffer before use, in order to obtain a solution at 0.25 IU/ml).

**Reagent 2 (R2): Thrombin (h):**

Purified human Thrombin, mainly in the  $\alpha$  form, lyophilized vial containing about 120 NIH (IU), or about 150 nkats (when determined in optimized conditions with CS-01 (38) specific substrate). ( $\alpha$ -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).

2 vials (each vial to be restored with 1 mL of distilled water, then diluted 1:5 in R4 buffer before use, in order to obtain a solution at about 30 nkats/ml).

**Reagent 3 (R3): Substrate:**

Chromogenic substrate specific for Thrombin (CS-01(38)), lyophilized vial of about 6.25  $\mu$ mol, in presence of mannitol.

2 vials (each vial to be restored with 5 mL of distilled water, in order to obtain a concentration of 1.25 mM).

**Reagent 4 (R4): Buffer:**

Assay reaction buffer Tris 0.05M, NaCl 0.175M, EDTA 0.0075M, at pH 8.40, containing Bovine Serum Albumin (BSA) at 0.2% and sodium azide as preservative.

4 vials of 25 ml, ready to use.

**Note:**

- Each donor unit of human plasma used for the purification of Antithrombin and Thrombin was tested and found negative for HIV antibodies, HBS Ag and HVC antibodies. However, no assay may warrant the total absence of infectious agents. Any product of human origin must then be handled with all the required cautions, as being potentially infectious.
- BSA was prepared from bovine plasma, which was tested for the absence of infectious agents, and collected from animals free from BSE. However, no test may totally exclude the absence of infectious agents. As any product of bovine origin, it must be used with all the cautions required for handling a material potentially infectious.
- The Thrombin and Antithrombin concentrations are adjusted, when required, for each lot, for providing the right reactivity in the assay.
- Sodium azide (0.9 g/l), contained in R4 buffer, may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.

**STORAGE CONDITIONS:**

Unopened reagents, must be stored at 2–8 °C, in their original packaging box. They are then stable until the expiration date printed on the label

**Note:** Stability studies for 3 weeks at 30°C show that the reagents can be shipped at room temperature for a short period without damage.

**PREPARATION AND STABILITY OF REAGENTS:**

**Note:** Reconstitution volumes can vary according to the automate used. Refer to each specific instrument adaptation.

**REAGENT 1: Human Antithrombin (AT)**

Reconstitute each vial with exactly 1 mL of distilled water. Shake thoroughly until complete dissolution of the contents (vortex). Let homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time. Just before use, dilute 1:5 with R4 buffer (if all the vial is used, add 4 ml of R4 buffer to the 1 ml of restored AT). Homogenize the contents before each use.

Stability of ATIII, restored with 1 ml, provided that any contamination or evaporation is avoided, kept in its original vial:

- 15 days at 2-8°C.
- 4 days at room temperature (18-25°C).
- 6 months frozen at -20°C or below. (Before use thaw in a water bath at 37°C for at least 15 min.).

**Cautions:** freezing conditions and stability of the thawed product should be checked in the working conditions of the laboratory user.

In our experience, ATIII diluted 1:5 in R4 is stable for about 8 hours at RT (18-25°C).

**REAGENT 2: Human Thrombin**

Reconstitute each vial with exactly 1 mL of distilled water. Shake thoroughly until complete dissolution of the contents (vortex). Let to homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time. Just before use, dilute 1:5 with R4 buffer (if all the vial is used, add 4 ml of R4 buffer to the 1 ml of restored thrombin). Homogenize the contents before each use.

Stability of Thrombin, restored with 1 ml, provided that any contamination or evaporation is avoided, kept in its original vial:

- 15 days at 2-8°C.
- 4 days at room temperature (18-25°C).
- 6 months frozen at -20°C or below. (Before use thaw in a water bath at 37°C for at least 15 min.).

**Cautions:** freezing conditions and stability of the thawed product should be checked in the working conditions of the laboratory user.

In our experience, Thrombin diluted 1:5 in R4 is stable for about 8 hours at RT (18-25°C).

**REAGENT 3: Thrombin specific chromogenic substrate**

Reconstitute each vial with exactly 5 mL of distilled water. Shake thoroughly until complete dissolution of the contents (vortex). Let to homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time (vortex). Check that all the substrate is dissolved before use.

Homogenize the contents before each use.

Stability of restored substrate, provided that any contamination or evaporation is avoided, kept in its original vial:

- 15 days at 2-8°C.
- 4 days at room temperature (18-25°C).
- 6 months frozen at -20°C or below. (Before use thaw in a water bath at 37°C for at least 15 min.).

**Cautions:** freezing conditions and stability of the thawed product should be checked in the working conditions of the laboratory user.

**REAGENT 4: Assay Reaction Buffer at pH 8.40**

Ready to use vial of 25 ml. Shake before use.

Stability of opened original vial:

- 1 month at 2-8°C.
- 7 days at room temperature (18-25°C).

Provided that no contamination of buffer occurs.

**Cautions:**

In order to improve stability, reagents must be closed with their original stoppers and screw caps following each use. Reagents must be handled with care, in order to avoid any contamination during use.

If the substrate becomes yellow, this indicates presence of a contaminant. It must be rejected, and a new vial must be used.

Incubating the reconstituted vials at RT allows stabilizing the reagents, and obtaining a homogeneous reactivity. Take care to limit as much as possible any evaporation of the reagents during use, eg. by using chimneys.

**Note:**

- The lyophilized vials (Reagents 1, 2 and 3) are closed under vacuum. Remove carefully the stopper, in order to avoid any loss of powder when opening the vials.
- According to the automated method used, the reagents can be reconstituted with volumes different from those recommended. In any case, the established reactive ratios (respective reagent concentrations in the reactive milieu) between the reagents must be strictly respected.
- Use only reagents from kits with a same lot number. Do not use reagents from kits with different lots when running the assay. Reagents are optimized for each lot of kits.

**REAGENTS REQUIRED BUT NOT PROVIDED:****Reagents:**

- Distilled water.
- Acetic acid (20%) or 2% citric acid (end point method).
- Heparin Reference Material (USP, International Standards from NIBSC, Internal References, etc.); alternatively commercially available lyophilized sets of UFH calibrators and controls in plasma or purified milieu, titrated for anti-IIa activity.

#### Materials:

- Spectrophotometer or automatic instrument for chromogenic assays.
- Stopwatch.
- Calibrated pipettes.

#### TESTED SPECIMEN:

**Purified:** dilute the heparin preparation with R4 buffer in order to bring it at a concentration within the assay working range (and preferably 0.005 to 0.040 IU/ml).

#### Plasma:

Blood (9 volumes) must be collected on 0.109 M citrate anticoagulant (1 volume), with great care, in order to avoid activation and PF4 release. Sampling must be performed through a net venipuncture, and the first drops must be discarded. Specific collection tubes for heparin testing, such as the CTAD (Citrate, Theophylline, Adenosine and Dipyridamole) tubes, can be used. They improve specimen stability.

- Within 1 hour, blood must be centrifuged at 3,000 g for 20 min at 18°C or below, and plasma decanted into a plastic tube, using a plastic pipette.
- Storage of plasma:
  - Up to 2 hours at 20°C
  - Up to 1 month frozen at -20°C or below (before use, thaw for 15 min. in a water bath at 37°C).

**Note:** Refer to GEHT or CLSI/NCCLS recommendations for further instructions on specimen collection, handling and storage. Discard any plasma presenting an unusual aspect (haemolysed, lipaemic aspect....).

#### CALIBRATION CURVE:

Prepare the calibration curve with commercial titrated preparations or by preparing your own calibrators.

Using the Heparin reference material, prepare a calibration curve of Heparin, in a normal human citrated plasma pool collected in order to avoid any platelet activation (for assaying plasma samples) or in R4 buffer (for assaying samples in purified milieu), as follows:

Heparin (IU/ml):	0.0	0.25	0.50	0.75	1.0
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Then prepare a 1:25 dilution (i.e. 0.1 ml + 2.4 ml) of each point with R4 buffer for the assay.

In order to get the full assay performances, the calibration curve must be prepared just before running the assay.

#### TESTED SAMPLES AND CONTROLS:

Plasma samples (expected concentrations  $\leq 1$  IU/ml) are tested at the 1:25 dilution in R4 buffer.

Samples in purified milieu are tested using an appropriate dilution in R4 buffer to get an expected tested concentration in the indicative range 0.005 to 0.04 IU/ml.

#### TEST PROCEDURE:

The Heparin Anti-IIa assay is specifically designed for two stage methods, automated on instruments, or used manually with end point methods.

The assay is performed at 37°C and the color developed is measured at 405 nm.

Whether 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 Heparin.

#### • Manual method:

Into the microwell or the plastic test tube, incubated at 37°C, introduce:

	Microwell	Test Tube
Reference material or tested sample (at the 1:25 or appropriate dilution).	40 $\mu$ l*	200 $\mu$ l
Antithrombin	40 $\mu$ l*	200 $\mu$ l
Mix and incubate at 37°C, for 2 minutes then introduce:		
Thrombin preincubated at 37°C	40 $\mu$ l*	200 $\mu$ l
Mix and incubate at 37°C, for exactly 2 minutes then introduce:		
Thrombin Substrate, preincubated at 37°C	40 $\mu$ l*	200 $\mu$ l
Mix and incubate at 37°C for exactly 1 minute		
Then stop the reaction by introducing		
Citric Acid (20g/L)	80 $\mu$ l**	400 $\mu$ l
Mix and measure the absorbance at 405nm against the corresponding blank.		

The yellow color is stable for 2 hours.

The sample blank, when required, is obtained by mixing the reagents in the reverse order from that of the test i.e.: Citric acid (20g/l), Thrombin substrate, Thrombin, Antithrombin and heparinized sample.

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

(Or \*50 $\mu$ l and \*\*100 $\mu$ l if preferred)

#### • Automated methods:

Adaptations to the various analysers (STA-R, etc...) are available upon request. Reconstitution volumes are susceptible to vary according to the automate used. Refer to each specific adaptation and specific cautions for each instrument.

**Note:** Unless an adaptation is duly validated, if higher or lower reactive volumes are required for the method used, the same respective proportions for each reagent concentration, and for the overall reactive volume, must be strictly respected, in order to keep a homogeneous reactivity.

#### QUALITY CONTROL:

Use of quality controls allows validating the homogeneous reactivity of the assay to heparin, from run to run, when using a same lot of reagents.

**Note:** A new calibration curve must be carried out for each new batch of reagents, after an important maintenance of the instrument, or if measured values are not in compliance with the one expected. Each laboratory can define its own acceptance range, according to the protocols and instruments used.

#### RESULTS:

For the manual end point method, using a lin-log graph paper, plot the heparin concentrations (0 to 0.04 IU/ml) on abscissa (Lin), and the corresponding A405 on ordinates (Log). Alternatively, statistics software can be used for establishing the dose response calibration curve. A semi-log inverse linear relationship is obtained between heparin concentrations and Absorbances (A405).

Draw the calibration curve obtained (with some instrument applications a lin-lin type curve can also be obtained).

Calculate the "r<sup>2</sup>" value. Calibration is acceptable if:

$$r^2 \geq 0.98$$

Usually, when using the manual test tube method, the A405 values range from about 1.70 (1.70  $\pm$  0.20) for the 0 IU/ml Heparin concentration, to about 0.50 (0.50  $\pm$  0.20) for the 0.04 IU/ml Heparin concentration in the assayed dilution.

Indicatively, for the microplate method, A405 is expected from about 1.20 (1.20  $\pm$  0.20) for the 0 IU/ml Heparin concentration, to about 0.30 (0.30  $\pm$  0.20) for the 0.04 IU/ml Heparin concentration in the assayed dilution.

A405 values can differ according to the instrument application used.

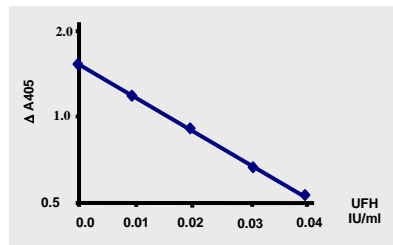
Deduce the heparin concentration for the tested specimen directly from the calibration curve (concentration corresponding to the measured A405), or by using the software.

Multiply the concentration measured by the specimen dilution factor (i.e. x25 for plasma).

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

#### EXAMPLE OF CALIBRATION CURVE:

The calibration curves herebelow, obtained with UFH, is indicated as an example only, using the manual test tube method. Only the calibration curve generated for the series of measures performed must be used.



#### ASSAY PERFORMANCES:

Dynamic range: 0.002 to 0.04 IU/ml in the tested dilution (ie 0 to 1 IU/ml in plasma)

Detection threshold: ~ 0.002 IU/ml in the tested dilution.

Standardization: International (NIBSC) or Internal reference for Unfractionated Heparin (UFH), or Pharmacopoeia preparations spiked in plasma.

In order to get the optimal assay performances, the working instructions must be carefully observed.

#### CHARACTERISTICS:

This heparin assay is a two stage assay for measuring accurately and sensitively heparin concentrations in plasma, or purified solutions. Tested sample needs to be diluted before assaying it.

Purified human thrombin used in the assay is mainly present in the  $\alpha$  form (obtained by direct activation of Prothrombin) which, for an equivalent concentration in chromogenic activity, exhibits a higher coagulant reactivity than "degraded"  $\beta$  or  $\gamma$  forms.

This assay, using a predilution of Antithrombin and Thrombin reagents in R4 buffer, is in compliance with the US Pharmacopoeia (USP36:2013) Anti-Factor IIa assay for unfractionated heparin.

This assay can also be adjusted by using a « Tris 0.05M, NaCl 0.15M, BSA1%, pH 7.4 » buffer (e.g. #AR005A/K) (instead of R4 buffer) to dilute Antithrombin (R1), Thrombin (R2) and samples, to fit the European Pharmacopoeia (EP:2013) recommended protocol.

#### VARIANT PROTOCOL:

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.

(\*) For UFH, 1 International Unit (IU) is equivalent to 1 USP Unit (2).

#### REFERENCES:

1. Van Putten J et al. Automated spectrophotometric heparin assays. Comparison of methods. Haemostasis, 14, 195-204, 1984.
2. USP Statement on Heparin Potency Unit Assignment and Harmonization with the international Standard for Unfractionated Heparin (21 Aug 2009)