BIOPHEN
HEPARIN ANTI-IIa (kinetics)

Ref: 221020

Kinetics/competitive method for the measurement of heparin, and heparin-like anticoagulants, in buffer, using an anti-IIa chromogenic assay

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

HYPHEN BioMed
Sales and Support:
CoaChrom Diagnostica GmbH
www.coachrom.com | info@coachrom.com
Tel: +43-1-236 222 1 | Fax: +43-1-236 222 111
Toll-free contact for Germany:
Tel: 0800-24 66 33-0 | Fax: 0800-24 66 33-3

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INTENDED USE:
This Heparin Anti-IIa method is a kinetics/competitive chromogenic assay for measuring the concentration of heparin, and heparin-like anticoagulants, using an automatic or a manual technique, on heparin concentration ranges from 0.0 to 6.0 IU/ml. This method is to be used only for testing heparin in buffer, or purified systems. **IT IS NOT APPROPRIATE FOR PLASMA.**
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 kinetics/competitive chromogenic anti-IIa assay developed for measuring heparin (UFH), in buffers or purified milieu.
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 Danaparoïd, inhibit more efficiently Factor Xa than thrombin. 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 kinetics/competitive method based on the inhibition of a constant amount of Thrombin (IIa), by the tested heparin in presence of exogenous antithrombin, and the simultaneous hydrolysis of a Thrombin specific chromogenic substrate, by remaining active Thrombin. 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 kit contains 2 vials of a specific Thrombin substrate, 2 vials of human Antithrombin (AT III), 2 vials of Human Thrombin, and 2 vials of assay reaction buffer.

Reagent 1 (R1):

Human Antithrombin (AT III), lyophilised:
2 vials (each vial to be restored with 5 mL of distilled water).

Reagent 2 (R2):

Chromogenic substrate specific for Thrombin, lyophilized in presence of mannitol.

2 vials (each vial to be restored with 10 mL of distilled water).

Reagent 3 (R3):

Human Thrombin, Lyophilised:
2 vials (each vial to be restored with 10 mL of distilled water).

Reagent 4 (R4):

Assay reaction buffer (Tris-NaCl-Na_2EDTA at pH 8.40), containing 1% BSA. 2 vials of about 20 ml, ready to use.

Note:

- The Human plasma used for the purification of Thrombin and Antithrombin 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, this BSA must be used with all the cautions required for handling a material potentially infectious.
- The Human Thrombin and Antithrombin concentrations are adjusted for each lot for providing the right reactivity in the assay,

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.

PREPARATION AND STABILITY OF REAGENTS:

REAGENT 1: Human Antithrombin (AT III)
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. Homogenize the contents before each use.
Stability of restored ATIII, provided any contamination or evaporation is avoided, kept in its original vial:

- 15 days at 2-8°C.
- 3 days at room temperature (18-25°C).
- 6 months frozen at -20°C or below.

REAGENT 2: THROMBIN SPECIFIC CHROMOGENIC SUBSTRATE

Reconstitute each vial with exactly 10 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. Homogenize the contents before each use. Stability of restored substrate, provided any contamination or evaporation is avoided, kept in its original vial:

- 15 days at 2-8°C.
- 3 days at room temperature (18-25°C).
- 6 months frozen at -20°C or below.

REAGENT 3: Human Thrombin
Reconstitute each vial with exactly 10 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. Homogenize the contents before each use. Stability of restored Thrombin, provided any contamination or evaporation is avoided, kept in its original vial:

- 15 days at 2-8°C.
- 3 days at room temperature (18-25°C).
- 6 months frozen at -20°C or below.

REAGENT 4: Assay Reaction Buffer at pH 8.40
Ready to use vial of 20 ml.
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.

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 thrombin and its substrate 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).
- Physiological Saline (9 g/L NaCl), and physiological saline containing 1% BSA (and/or 1% PEG6000).
- Heparin Reference Material (USP, International Standards from NIBSC, Internal References, etc.); alternatively commercially available lyophilized sets of UFH calibrators and controls in purified milieu, titrated for anti-IIa activity.

Materials:

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

Calibration curve:

Using the Heparin reference material, prepare a calibration curve of Heparin in physiological saline (9 g/L NaCl) containing 1% BSA, as follows:

Heparin (IU/ml):	0.0	0.5	1.0	2.0	4.0	6.0

TEST PROCEDURE:

The Heparin Anti-IIa (kinetics) assay is specifically designed for Kinetics/competitive 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 test tube, incubated **at 37°C**, introduce:

	Microwell	Test Tube
Reference or tested Heparin solution.	20 µl	100 µl
Antithrombin (200 µg/ml)	20 µl	100 µl
Assay Reaction Buffer	100 µl	500 µl
Thrombin Substrate	40 µl	200 µl
Mix and incubate at **37°C**, for 2-3 minutes then introduce:		
Human Thrombin **Preincubated at 37°C**	40 µl	200 µl
Mix and incubate at **37°C for exactly,**	5 minutes.	5 minutes.
Then stop the reaction by introducing		
Citric Acid (20g/L)	80 µl	400 µl
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 opposite order from that of the test i.e., Citric acid (20g/l)**,** Thrombin, Thrombin substrate, reaction buffer, Antithrombin and heparin solution.
Measure the absorbance **at 405 nm**. The sample blank value must be deduced from the absorbance measured for the corresponding assay.

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 UFH, 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 concentration (0.0 to 6.0 IU/ml) on abscissa (Lin), and the corresponding A405 on ordinate (Log). Alternatively, statistics software can be used for establishing the dose response calibration curve. A semi-log inverse linear relationship is obtained between heparin concentration and Absorbance (A405).
Draw the calibration curve obtained.
Calculate the "r^2" value. Calibration is acceptable if:

$$r^2 \geq 0.98$$

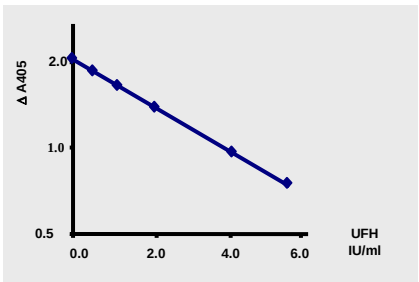
Usually, when using the manual test tube method, the A405 values range from about 2.00 (2.00 ± 0.20) for the 0.0 IU/ml Heparin concentration, to about 0.80 (0.80 ± 0.20) for the 6.0 IU/ml Heparin concentration. Indicatively, for the microplate method, A405 is expected from about 1.50 (1.50 ± 0.20) for the 0.0 IU/ml Heparin concentration, to about 0.60 (0.60 ± 0.20) for the 6.0 IU/ml Heparin concentration. 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.

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

EXAMPLE OF CALIBRATION CURVE:

The calibration curve here below, 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.



CHARACTERISTICS:

This kinetics method is based on the simultaneous inhibitory action of ATIII (in excess) complexed with heparin (limiting factor), which inhibits thrombin, and proteolysis of the specific thrombin substrate by residual thrombin.
The substrate concentration is adjusted in order to allow the obtaining of a dynamic range from 0.0 to 6.0 IU/ml heparin concentration.
This approach permits testing heparin concentrations up to 6.0 IU/ml in the tested solution without requiring any further dilution step.

ASSAY DETECTION LIMIT:

≤ 0.20 IU/ml

APPLICATIONS:

Measurement of the specific anti-IIa activity of heparin and heparin-like anticoagulants, in purified milieu, using a kinetics / competitive assay.

REFERENCES:

Leslie B et al. Investigation of the anticoagulant mechanism of a covalent antithrombin-heparin complex J Biol Chem 52 (273): 34730-34736 (1999).