

## 5-TEST USP-UFH Anti-IIa Heparin Kit in compliance with US Pharmacopoeia

**REF** 5D-50457

*Complete set of individual reagents for the measurement of heparin in buffer using an anti-FIIa chromogenic assay for pharmaceutical preparations in compliance with US Pharmacopoeia.*

**For Research Use Only.  
Not for Use in Diagnostic Procedures.  
Mixed storage.**

### INTENDED USE

This Heparin Anti-FIIa method can be used as an endpoint or kinetic chromogenic assay for measuring the concentration of heparin in the range of 0.005-0.030 USP Heparin Units/mL (IU/mL). The method is to be used for anti-FIIa activity of Unfractionated Heparin following the recommendations of the US Pharmacopoeia.

### TEST PRINCIPLE

Heparin is a sulphated polysaccharide with a high affinity for antithrombin. Antithrombin complexed with heparin has a fast and potent inhibitory activity for coagulation factors IXa, Xa and FIIa (Thrombin). The Heparin Anti-FIIa method is a method based on the inhibition of a constant amount of Thrombin (FIIa) by the tested molecule in presence of exogenous antithrombin and the simultaneous hydrolysis of a thrombin-specific chromogenic substrate by remaining active thrombin. The colour is then read photometrically at 405 nm. There is an inverse relationship between the heparin concentration and colour development measured at 405 nm.

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

### REAGENTS INCLUDED

#### pH 8.4 Buffer (5-BUFFER 5D-80434)

Tris-NaCl-EDTA-PEG-6000 Buffer salts pH 8.4  
0.050 M Tris buffer pH 8.4 at 25°C, 0.175 M NaCl, 0.0075 M EDTA, 0.10% (w/v) PEG-6000

**Kit content:** 1 Pouch

**Reconstitution:** dissolve pouch content in 1000 mL distilled water.

**Buffer stability after reconstitution:** 4 weeks at 2-8°C when protected from any contamination.

#### Thrombin (Human)

Lyophilized Human Thrombin

**Kit content:** 3 Vials, 12 IU per vial

**Reconstitution:** dissolve vial content in 0,60 mL distilled water

**Stock concentration:** 20 IU/mL

**Working concentration:** 5 IU/mL (stock solution is then diluted 1:4 in 5-BUFFER 5D-80434)\*

**Reagent stability after reconstitution, excluding any contamination or evaporation, and stored in the original vial, is:**

- 72 h at 2-8°C.
- 24 h at room temperature (18-25°C).
- 6 months frozen at -20°C or below\*\*

#### Antithrombin (Human)

Lyophilized Human Antithrombin III Reagent

**Kit content :** 1 Vial, 4 IU per vial

**Reconstitution:** dissolve vial content in 0,8 mL distilled water

**Stock concentration:** 5 IU/mL

**Working concentration:** 0.125 IU/mL (stock solution diluted 1:40 in 5-BUFFER 5D-80434)

**Reagent stability after reconstitution, excluding any contamination or evaporation, and stored in the original vial, is:**

- 72 h at 2-8°C.
- 24 h at room temperature (18-25°C).
- 6 months frozen at -20°C or below\*\*

#### Chromogenic Thrombin Substrate

Lyophilized Chromogenic Substrate for Thrombin: D-Phe-Pip-Arg-pNA

**Kit content:** 1 Vial, 12,5 mg per vial (approx. 20 µmol/vial)

**Reconstitution:** dissolve vial content in 4 mL distilled water

**Stock concentration:** 5mM

**Working concentration:** 1.25 mM (stock solution diluted 1:4 in distilled water)

**Reagent stability after reconstitution, excluding any contamination or evaporation, and stored in the original vial, is:**

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

### STORAGE CONDITIONS:

Unopened reagents must be stored in their original packaging at their labelled temperature. They are then stable until the expiration date printed on the label.

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

\* Alternatively, for using all reagents in a single run, Thrombin can be restored directly in its vial with 0.60 ml distilled water then 1.80 ml of 5-Buffer.

\*\*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.

### OTHER REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:

#### Reagents:

- Distilled water
- Acetic acid 20 % V/V
- USP, EP or International Standards from NIBSC, Internal Reference preparations

#### Materials:

- Spectrophotometer or automatic instrument for chromogenic assays
- Stopwatch
- Calibrated pipettes
- Water bath or heating block
- Plastic tubes or 96 well microplates

### TEST PROCEDURE

Prepare at least 4 dilutions of your Reference Heparin preparation in 5-BUFFER 5D-80434 in the concentration range 0.005-0.030 USP Heparin Units/mL (IU/mL)

Prepare at least dilutions of your sample in 5-BUFFER 5D-80434 to obtain heparin concentrations similar to those of the reference dilutions.

Use 5-BUFFER 5D-80434 as a blank to monitor the behaviour of the reagents during the experiment. Add a blank before and after each series of reference or sample dilutions.

All reference and sample dilutions should be tested at least in duplicate.

## ASSAY PROTOCOL:

Add **200 µL** of **Antithrombin III** solution to a tube with **100 µL** of reference dilution, sample dilution or blank. Mix gently and incubate **60 seconds at 37°C** in a water bath or heating block.

Add **50 µL** of **Human Thrombin** solution and incubate **60 seconds at 37°C**.

Add **100 µL** of **FIIa Chromogenic Substrate** and incubate at **37°C**.

Stop the reaction after **exactly 4 minutes** with **100 µL acetic acid solution**.

Measure the absorbance at 405 nm or measure the absorbance change per minute at 405nm. If necessary, adjust the incubation time to give best dose-response curve.

The relative standard deviation (RSD) over the blank readings should be less than 10%.

Plot the log absorbance versus heparin concentrations in USP Heparin Units/mL (IU/mL). Determine the slope for the regression line of both reference and sample curves to calculate the potency. Follow statistical analysis of results of biological assays and tests in compliance with US Pharmacopoeia guidelines for slope ratio or parallel-line assays.

### Tube Method

Reagent	Volume
Antithrombin III 0.125 IU/mL preheated at 37°C	200 µL
Reference, test sample or blank	100 µL
Mix and incubate for at least 1 minute at 37°C	
Human Thrombin 5 IU/mL preheated at 37°C	50 µL
Mix and incubate for 1 minute at 37°C	
Chromogenic substrate 1.25 mM preheated at 37°C	100 µL
Mix and incubate at 37°C exactly for 4 minutes* Stop the reaction by adding:	
Acetic acid 20%	100 µL
Mix and measure the absorbance at 405 nm	

\*Or start measuring  $\Delta OD_{405nm}/min$  (kinetic method)

### ALTERNATIVE METHOD

The assay can be miniaturized in 96 wells microplate.

### Microplate Method

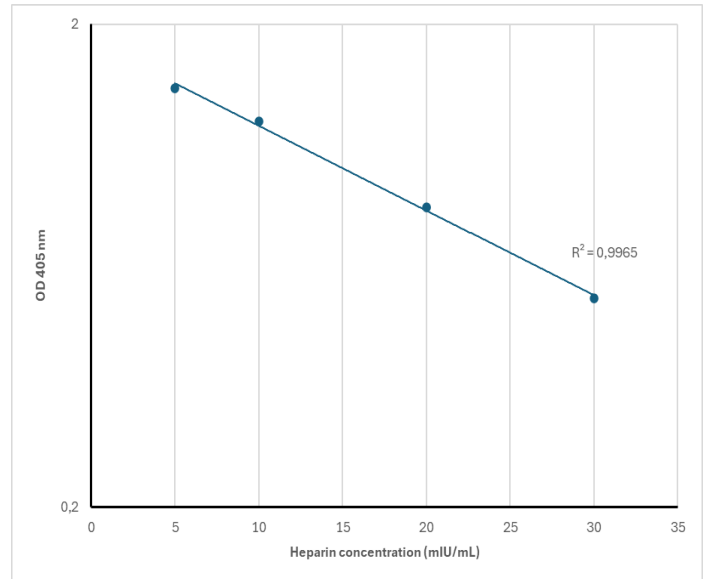
Reagent	Volume
Antithrombin III 0.125 IU/mL preheated at 37°C	100 µL
Reference, test sample or blank	50 µL
Mix and incubate for at least 1 minute at 37°C	
Human Thrombin 5 IU/mL preheated at 37 °C	25 µL
Mix and incubate for 1 minute at 37°C	
Chromogenic substrate 1.25 mM preheated at 37°C	50 µL
Mix and incubate at 37°C exactly for 4 minutes* Stop the reaction by adding:	
Acetic acid 20%	50 µL
Mix and measure the absorbance at 405 nm.	

\*Or start measuring  $\Delta OD_{405nm}/min$  (kinetic method)

Application protocols for automated analysers are available from [info@5-diagnostics.com](mailto:info@5-diagnostics.com).

### Example of calibration curve:

The calibration curve below is indicated only as an example. The calibration curve established for each series of testing must be used for measuring the heparin concentrations.



### ASSAY DETECTION RANGE

0.005-0.030 USP Heparin Units/mL (IU/mL)

### APPLICATIONS

Measurement of the specific anti-FIIa activity of heparin and heparin-like anticoagulants in purified milieu using a two-stage assay. This procedure is in compliance with the quality control of Unfractionated Heparin preparations listed in US Pharmacopoeia.

### REFERENCES

USP 40(208) Anti-Factor Xa and Anti-Factor IIa Assays for Unfractionated and Low Molecular Weight Heparins



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