

## 5-TEST EP-LMWH Anti-IIa starter set in compliance with European Pharmacopoeia

**REF** 5D-90451

*Complete set of individual reagents for the measurement of heparin and heparin-like anticoagulants in buffer using an anti-FIIa chromogenic assay for pharmaceutical preparations in compliance with Eur. 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 0.015-0.075 IU/mL. This method is to be used for anti-FIIa activity of Low Molecular Weight Heparin following the recommendations of the European 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 IIa (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 concentration of heparin 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:

#### 5-BUFFER USP/Ph.Eur. Tris-NaCl-BSA Buffer salts pH 7.4

Ref. 5D-80433

5-BUFFER USP/Ph.Eur. Tris-NaCl-BSA Buffer salts pH 7.4, 1000 mL 0.050 M Tris Buffer pH 7.4 at 25°C, 0.150 M NaCl, 1.0% (w/v) BSA (bovine serum albumin).

**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.

#### 5-BUFFER USP/Ph.Eur. Tris-NaCl-EDTA Buffer salts pH 8.4

Ref. 5D-80431

5-BUFFER USP/Ph.Eur. Tris-NaCl-EDTA Buffer salts pH 8.4, 500 mL 0.050 M Tris Buffer pH 8.4 at 25°C, 0.175 M NaCl, 0.0075 M EDTA

**Kit content:** 1 Pouch

**Reconstitution:** dissolve pouch content in 500 mL distilled water.  
**Buffer stability after reconstitution:** 4 weeks at 2-8°C when protected from any contamination.

### 5-ENZYME Thrombin (Human)

Ref. 5D-60230

Lyophilized Human Thrombin

**Kit content:** 2 Vials, 100 IU per vial

**Reconstitution:** dissolve vial content in 2 mL distilled water

**Stock concentration:** 50 IU/mL

**Working concentration:** 5 IU/mL (stock solution diluted 1:10 in 5-BUFFER 5D-80433)

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

- 21 days at 2-8°C.
- 7 days at room temperature (18-25°C).
- 6 months frozen at -30°C or less\*

### 5-PROTEIN Antithrombin (Human)

Ref. 5D-60104

Lyophilized Human Antithrombin III Reagent

**Kit content:** 1 Vial, 10 IU per vial

**Reconstitution:** dissolve vial content in 5 mL distilled water

**Stock concentration:** 2 IU/mL

**Working concentration:** 0.5 IU/mL (stock solution diluted 1:4 in 5-BUFFER 5D-80433)

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

- 1 month at 2-8°C.
- 72 hours at room temperature (18-25°C).
- 6 months frozen at -20°C or less\*

### 5-CHROM-38 Chromogenic Factor IIa Substrate

Ref. 5D-30805

**Sequence:** D-Phe-Pip-Arg-pNA

**Kit content:** 1 Vial, 25 mg per vial/ 45 µmol/vial

**Reconstitution:** dissolve vial content in 15 mL distilled water

**Stock concentration:** 3 mM

**Working concentration:** 0.5 mM (stock solution diluted 1:6 in 5-BUFFER 5D-80431).

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

- 3 months at 2-8°C.
- 7 days at room temperature (18-25°C).
- Do not freeze.

### STORAGE CONDITIONS:

Unopened reagents must be stored in their original packaging at 2-8°C. 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.

\*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
- Glacial 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
- Calibrated water bath or heating block
- Plastic tubes or 96 well microplates

#### TEST PROCEDURE:

Prepare 4 independent calibration curves of minimum 4 points spanning 0.015 to 0.075 IU/mL of your reference Heparin Preparation in Buffer 5D-80433. Use Buffer 5D-80433 as a blank for the reaction. Prepare 4 independent dilutions of your sample in Buffer 5D-80433.

Add 50 µL of 5-PROTEIN Antithrombin (Human) to 50 µL of sample or calibrator or blank. Mix gently and incubate 60 seconds at 37°C in a water bath or heating block.

Add 100 µL of Human Thrombin solution and incubate 60 seconds at 37°C.

Add 250 µL of pre-warmed 5-CHROM Chromogenic Factor IIa Substrate solution and incubate exactly for 240 seconds at 37°C.

Stop the reaction with 375 µL acetic acid solution.

Measure the absorbance at 405 nm.

Plot the absorbance versus log of heparin concentrations in International Units/mL.

If necessary adjust the incubation time to give best dose-response curve.

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 Pharmacopoeia guidelines for parallel-line assays.

Reagent	Tube
Antithrombin III 0.5 IU/mL	50 µL
Reference, test sample or blank	50 µL
Mix and incubate for 1 minute at 37°C	
Human Thrombin 5 IU/mL	100 µL
Mix and incubate for 1 minute at 37°C	
Chromogenic substrate 0.5 mM pre-warmed at 37°C	250 µL
Mix and incubate at 37°C exactly for 4 minutes Stop the reaction by adding:	
Acetic acid 20%	375 µL
Mix and measure the absorbance at 405 nm against the corresponding blank.	

#### ALTERNATIVE METHODS

The assay can be miniaturized in 96 wells microplate.

Reagent	Tubes
Antithrombin III 0.5 IU/mL	25 µL
Reference, test sample or blank	25 µL
Mix and incubate for 1 minute at 37°C	
Human Thrombin 5 IU/mL	50 µL
Mix and incubate for 1 minute at 37°C	
Chromogenic substrate 0.5 mM pre-warmed at 37°C	125 µL
Mix and incubate at 37°C exactly for 4 minutes Stop the reaction by adding:	
Acetic acid 20%	25 µL
Mix and measure the absorbance at 405 nm against the corresponding blank.	

Note: Reagent concentrations may be adapted in order to obtain higher OD values. Please contact [info@5-diagnostics.com](mailto:info@5-diagnostics.com).

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

#### ASSAY DETECTION RANGE

0.015-0.075 IU/mL

#### APPLICATIONS

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

#### REFERENCES

European Pharmacopoeia 8.0: 01/2014:0828 Assay of LMWH Heparin.



5-Diagnostics AG  
Heuberg 7, 4051 Basel,  
Switzerland  
Tel: +41 61 588 07 84  
[www.5-diagnostics.com](http://www.5-diagnostics.com)  
[info@5-diagnostics.com](mailto:info@5-diagnostics.com)