

## 5-TEST USP-LMWH Anti-Xa starter set in compliance with US Pharmacopoeia

**REF** 5D-90456

*Complete set of individual reagents for the measurement of heparin and heparin-like anticoagulants in aqueous solutions using an anti-FXa 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-FXa method can be used as an endpoint or kinetic chromogenic assay for measuring the concentration of heparin in the range of 0.025-0.2 IU/mL. The method is to be used for the determination of anti-FXa activity of 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 IIa (Thrombin). FXa in excess, is neutralized in proportion to the amount of heparin (Heparin - AT- complex). The remaining amount of FXa hydrolyses the chromogenic substrate and liberates the chromophoric group pNA. 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.] + [FXa (excess)] → [FXa-AT-Hep.] + [residual FXa]

[residual FXa] + Substrate → Peptide + pNA

### REAGENTS INCLUDED

#### 5-BUFFER USP Tris-NaCl-PEG-6000 Buffer salts pH 7.4

Ref. 5D-80432

5-BUFFER USP Tris-NaCl-PEG-6000 Buffer salts pH 7.4, 1000 mL  
0.050 M Tris buffer pH 7.4 at 25°C, 0.150 M NaCl, 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.

#### 5-BUFFER USP Tris-NaCl Buffer salts pH 7.4

Ref. 5D-80430

5-BUFFER USP Tris-NaCl Buffer salts pH 7.4, 1000 mL  
0.050 M Tris buffer pH 7.4 at 25°C, 0.150 M NaCl

**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 Factor Xa (Bovine)

Ref. 5D-60217

Lyophilized Bovine FXa

**Kit content:** 1 Vial, 30 µg/vial lyophilized bovine FXa, stabilizers

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

**Stock concentration:** 15 µg/mL

**Working concentration:** 3 µg/mL (stock solution diluted 1:5 in 5-BUFFER 5D-80432). Concentration may be adopted as requested.

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

- 3 months at 2-8°C.
- 7 days at room temperature (18-25°C).
- 6 months frozen at -20°C or less.\*

#### 5-PROTEIN Antithrombin (Human)

Ref. 5D-60104

Lyophilized Human Antithrombin III Reagent

**Kit content:** 2 Vials, 10 IU per vial lyophilized human antithrombin, stabilizers. Specific activity >6 IU/mg

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

**Stock concentration:** 5 IU/mL

**Working concentration:** 0.5 IU/mL (stock solution diluted 1:5 in 5-BUFFER 5D-80432)

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

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

#### 5-CHROM-65 Chromogenic Factor Xa Substrate

Ref. 5D-30807

Chromogenic Substrate for Factor Xa: Z-D-Arg-Gly-Arg-pNA:2HCI

**Kit content:** 1 Vial with 25 mg (39 µmol/vial) synthetic chromogenic Factor Xa Substrate, highly purified and stabilized. Mannitol is added as a bulking agent.

**Reconstitution:** dissolve vial content in 13 mL distilled water

**Stock concentration:** 3 mM

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

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

- 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 0.025 IU/mL to 0.2 IU/mL of your reference Heparin Preparation in 5-BUFFER 5D-80432. Use 5-BUFFER 5D-80432 as a blank for the reaction.

Prepare 4 independent dilutions of your sample in 5-BUFFER 5D-80432.

Add 50 µL of preheated Antithrombin III solution 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 preheated Bovine Factor Xa solution and incubate 60 seconds at 37°C.

Add 250 µL of preheated FXa Chromogenic 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. In some cases log transformation of absorbance may be needed to obtain linearity.

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 US Pharmacopoeia guidelines for parallel-line assays.

Reagent	Tubes
Antithrombin III 1 IU/mL preheated at 37°C	50 µL
Reference, test sample or blank	50 µL
Mix and incubate for 1 minute at 37°C	
Bovine Factor Xa 3 µg/mL preheated at 37°C	100 µL
Mix and incubate for 1 minute at 37°C	
Chromogenic substrate 0.5 mM preheated 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	Microplate
Antithrombin III 1 IU/mL preheated at 37°C	25 µL
Reference, test sample or blank	25 µL
Mix and incubate for 1 minute at 37°C	
Bovine Factor Xa 3 µg/mL preheated at 37°C	50 µL
Mix and incubate for 1 minute at 37°C	
Chromogenic substrate 0.5 mM preheated 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.	

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

#### ASSAY DETECTION RANGE

0.025-0.2 IU/mL

#### APPLICATIONS

Measurement of the specific anti-FXa 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 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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