5-Kit USP/EP-UFH Anti-Xa Kit

For the assay of Unfractionated Heparin (UFH) in compliance with EP and USP

REF 5D-50460

Complete kit for the measurement of heparin and heparin-like anticoagulants in buffer using an anti-FXa chromogenic assay for pharmaceutical preparations, in compliance with EP and USP

For Research Use Only.
Not for Use in Diagnostic Procedures.

INTENDED USE

This Heparin Anti-FXa method can be used as an endpoint or kinetic chromogenic assay for measuring the concentration of heparin and heparin-like anticoagulants in a concentration range from 0.030-0.375 USP Heparin Units/mL (IU/mL). The method is to be used for the anti-FXa activity of Unfractionated Heparin following the recommendations of the European and US Pharmacopoeias.

TEST PRINCIPLE

Heparin is a sulphated polysaccharide with a high affinity for antithrombin (AT). AT complexed with heparin has a fast and potent inhibitory activity for coagulation factors IXa, Xa and IIa (Thrombin). The Heparin Anti-F-Xa method is based on the inhibition of a constant amount of FXa by the tested molecule in presence of exogenous AT, then hydrolysis of a thrombin-specific chromogenic substrate by remaining active FXa. The absorbance, read photometrically at 405 nm, 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

pH 8.4 buffer (5-Buffer 5D-80434)
Tris-NaCl-EDTA-PEG-6000 Buffer: 0.050 M Tris , 0.175 M NaCl, 0.0075 M EDTA, 0.10% (w/v) PEG-6000; pH 8.40 at 25°C; contains 0.1 g/L Sodium Azide (NaN3) as preservative§.
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.
This buffer is available separately under reference 5D-80434
(5-BUFFER USP/Ph.Eur. Tris-NaCl-EDTA-PEG-6000 Buffer salts)

§Caution: Sodium azide (NaN3), may react with lead and copper plumbing to form highly explosive metal azides. To avoid this risk, flush with large volumes of water when discarding into a sink.

Factor Xa (Bovine)
Lyophilized Bovine FXa
Kit content: 2 Vials, 20 µg per vial
Reconstitution: dissolve vial content in 2 mL distilled water
Stock concentration: 10 µg/mL
Working concentration: approximately 2.5 µg/mL (stock solution diluted 1:4 in 5-BUFFER 5D-80434); the exact concentration is adjusted for obtaining the right assay reactivity*.
Concentration may be adopted as requested.
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
Kit content: 2 Vials, 4 IU per vial
Reconstitution: dissolve vial content in 1 mL distilled water.
Stock concentration: 4 IU/mL
Working concentration: 1 IU/mL (stock solution 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**.

Factor Xa Chromogenic Substrate
Lyophilized Chromogenic Substrate for Factor Xa**:** Z-D-Arg-Gly-Arg-pNA·2HCl
Kit content: 2 Vials with 6 mg (~8 µmol/vial) synthetic chromogenic Factor Xa Substrate, highly purified and stabilized. Mannitol is added as a bulking agent.
Reconstitution: dissolve vial content in 8 mL water
Working concentration: 1 mM
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 2–8°C. When protected from any contamination, these are 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, FXa can be restored directly in its vial with 2 ml distilled water then 6 ml of 5-Buffer, and AT with 1 ml distilled water and 3 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 (alternatively 2% citric acid can be used)
- 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

USP TEST PROCEDURE

Prepare at least 5 dilutions of your reference Heparin Preparation spanning the concentration range from 0.030 to 0.375 USP Heparin Units/mL (IU/mL) in pH 8.4 Buffer.

Prepare 5 dilutions of your sample in 5-BUFFER 5D-80434 to have approximately the same activity equal to those of the Standard solution.

Perform the test with each Standard and Sample dilution in duplicate in suitable plastic tubes in a water bath or heating block set at 37°C.

EP TEST PROCEDURE

Prepare at least 4 dilutions of your reference Heparin Preparation spanning the concentration range from 0.030 to 0.375 USP Heparin Units/mL (IU/mL) in pH 8.4 Buffer.

Prepare 4 dilutions of your sample in 5-BUFFER 5D-80434 to have approximately the same activity equal to those of the Standard solution.

Perform the test with each Standard and Sample dilution in quadruplicate in suitable plastic tubes in a water bath or heating block set at 37°C.

Add a blank at the beginning and at the end of the procedure by replacing sample or reference with buffer. The 2 blank values should not differ significantly.

ASSAY PROTOCOL:

Add to each tube **120 µL** of **5-BUFFER 5D-80434** and then separately add **30 µL** of the different dilutions of **reference and samples**.

Add **150 µL** of preheated **Antithrombin (1 IU/ml)** solution to each tube, mix gently and incubate **120 seconds at 37°C**.

Add **300 µL** of preheated **Bovine Factor Xa (2.5 µg/ml)** solution and incubate **exactly 120 seconds at 37°C**.

Add **300 µL** of preheated **FXa Chromogenic Substrate (1 mM)** solution and incubate for **exactly 120 seconds at 37°C**. If necessary, adjust the incubation time to give best dose-response curve.

Stop the reaction with **150 µL** acetic acid solution.

Prepare a Blank for zeroing the spectrophotometer by adding the reagents in reverse order starting with the acetic acid and ending with 150 µL of 5-BUFFER 5D-80434.

Measure the absorbance at 405 nm.

Plot the log of the absorbance versus heparin concentrations in 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 assays.

The anti-Xa activity of the sample is calculated with the formula:

Result = A x (Slope Sample Curve/Slope Reference Curve)

Where A is the potency of the Reference preparation used.

Test Tube Method

Reagent	Volume
Buffer	**120 µL**
Diluted Reference or Sample	**30 µL**
Antithrombin 1 U/mL preheated at 37°C	**150 µL**
Mix and incubate for 2 minutes at 37°C	
Bovine Factor Xa 2.5 µg/mL preheated at 37°C	**300 µL**
Mix and incubate for 2 minutes at 37°C	
Chromogenic substrate 1 mM preheated at 37°C	**300 µL**
Mix and incubate for 2 minutes at 37°C **Stop the reaction by adding:**	
Acetic acid 20%	**150 µL**
Mix and measure the absorbance at 405 nm Using Log-Lin coordinates, plot the heparin concentrations (IU/ml) on abscissae, and the corresponding OD405 on ordinates and draw the calibration curve.	

ALTERNATIVE METHODS

The assay can be miniaturized in 96 wells microplate.

Dilute the prepared reference and sample dilutions further 1:5 with Buffer. For example, add 120 µL of 5-BUFFER 5D-80434 to 30 µL of these dilutions.

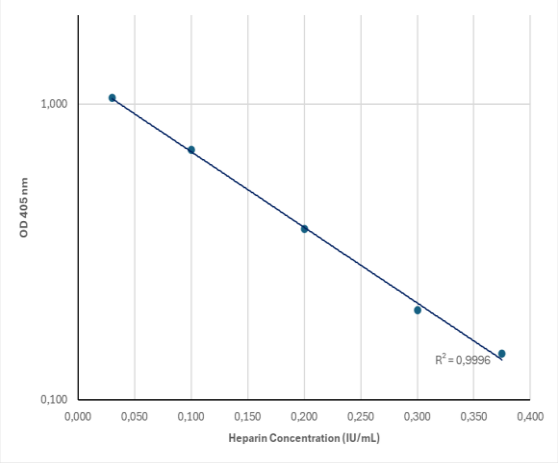
Microplate Method

Reagent	Volume
1:5 Diluted Reference or Sample dilutions	**40 µL**
Antithrombin 1 U/mL preheated at 37°C	**40 µL**
Mix and incubate for 2 minutes at 37°C	
Bovine Factor Xa 2.5 µg/mL preheated at 37°C	**80 µL**
Mix and incubate for exactly 2 minutes at 37°C	
Chromogenic substrate 1 mM preheated at 37°C	**80 µL**
Mix and incubate for 2 minutes at 37°C **Stop the reaction by adding:**	
Acetic acid 20%	**20 µL**
Mix and measure the absorbance at 405 nm	

Application protocols for automated analysers are available from 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

0.030-0.375 USP Heparin Units/mL (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 following the quality control of Unfractionated Heparin preparations listed in European and US Pharmacopoeias.

REFERENCES

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

European Pharmacopoeia 2.7.5 Assay of Heparin

5-DIAGNOSTICS

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