KRISHZYME®
Enoxaparin Factor Xa Assay kit

REF : KBBA04E

Ver 4.5

(200 test)

RUO

Chromogenic assay for testing Enoxaparin in purified systems by measurement of factor Xa inhibition, in compliance with pharmacopoeias (EP/BP) and FDA guidelines

RUO	**For Research Use Only**	REF	**Catalog Number**
	re At	LOT	**Batch Code**
	nufactured By		**logical Risk**
	iry Date		**nsult Operating Instructions**

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REF **KBBA04E** Σ **200 tests**

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Intended Use:

Enoxaparin Factor Xa is a chromogenic assay intended for the quantitative determination of enoxaparin in purified solutions by measurement of factor Xa inhibition activity. The kit can be used for 100 test reactions as per microtiter plate protocol.

Principle:

The inhibitory effect of anti-thrombin III (AT-III) on thrombin, factor Xa and other coagulation serine proteases in plasma is increased several thousand-fold by enoxaparin. This inhibition accounts for the anticoagulant effect of enoxaparin. The quantitative determination of enoxaparin levels by the measurement of their anti-Xa activity is a necessary tool for monitoring treatment efficacy.
Presence of Enoxaparin catalyzes the reaction between AT-III and Factor Xa. The factor Xa inhibition test is the most useful assay covering the widest variety of enoxaparin preparations. In the assay, the rate of factor Xa inhibition is directly proportional to the enoxaparin concentration since both factor Xa and AT-III are in excess. The residual factor Xa activity is inversely proportional to the enoxaparin concentration.

Materials Provided:

1. Human Anti-thrombin III Reagent (lyophilized) - 2 Vials
2. Bovine Factor Xa Reagent (lyophilized) - 2 Vials
3. Chromogenic Substrate (lyophilized) - 2 Vials
4. Instruction Manual

Materials to be provided by the End-User:

1. Microplate Reader / Spectrophotometer able to measure absorbance at 405 nm.
2. Adjustable pipettes to measure volumes ranging from 25 ul to 2500 ul, duly calibrated.
3. Deionized (DI) water.
4. Parallel line software for data analysis.
5. Plastic tubes or cuvettes or microtiter plates with overflow capacity ≤ 350 ul/well.
6. 37°C water bath or dry bath.
7. Timer/Stop watch.
8. Glacial Acetic Acid.
9. Absorbent paper.
10. Dilution Buffer.
11. Standard.

Storage and Stability Information:

Unreconstituted (lyophilized) reagents are stable until the expiration date indicated on the label when stored at 2-8°C.

1. **Human Anti-thrombin III Reagent:** Reconstituted reagent is stable for 2 weeks at 2-8°C and for 4 months at -20°C.

2. **Bovine Factor Xa Reagent:** Reconstituted reagent is stable for 2 weeks at 2-8°C and for 4 months at -20°C.

3. **Chromogenic Substrate:** Reconstituted reagent is stable for 2 weeks at 2-8°C and for 4 months at -20°C.

4. **Dilution Buffer** and **Acetic acid** are to be freshly prepared, prior to use.

Health Hazard Warnings:

1. The source material for the human anti-thrombin III has been found to be non-reactive for Hepatitis B Surface Antigen (HBsAg), Hepatitis C Virus (HCV) and Human Immunodeficiency Virus Type 1 and Type 2 (HIV-1, HIV-2) using FDA approved methods.

2. The enoxaparin (anti-FXa) anti-thrombin III reagent contains sodium azide that may react with lead or copper plumbing to form highly explosive azides.

Specimen Collection and Handling:

Purified Samples: Dilute the enoxaparin preparation with Dilution Buffer in order to bring it at a concentration within the assay working range.

Reagent Preparation:

Note: 1) Bring all reagents to room temperature.
2) All reagents should be diluted immediately prior to use.

1. **Human Anti-thrombin III Reagent:**
   Anti-thrombin III is a lyophilized preparation. For preparing working Solution reconstitute in 2.5 ml of Factor buffer pH 7.4 and leave for 15 minutes before running the experiment and use directly.

2. **Bovine Factor Xa Reagent:**
   Bovine Factor Xa (BFXA) Reagent is a lyophilized preparation For Reconstitution, add 5 ml of Factor buffer pH 7.4 and leave for 15 minutes before running the experiment. For preparing working solution add 800 ul of reconstituted BFXA reagent to 200 ul of Factor buffer pH 7.4.

3. **Chromogenic Substrate:**
   Chromogenic Substrate is a lyophilized substrate specific for Factor Xa activity. For Reconstitution, add 5 ml of Factor buffer pH 8.4 and leave it to stand for 15 minutes. For preparing working solution add 500 ul of reconstituted Chromogenic Substrate to 500 ul of Factor buffer pH 8.4.

4. **Dilution Buffer: For Standard / Sample** (Not provided in the kit):
   To be prepared with 50 mM Tris, 150 mM NaCl Adjust the pH upto 7.4.

5. **Dilution Buffer: For Chromogenic Substrate**(Not provided in the kit):
   Dissolve 6.10 g of tris (hydroxymethyl) aminomethane, 10.20 g of sodium chloride, 2.80 g of EDTA sodium, and, if suitable, between 1% of macrogol 6000 and/or 2.00 g of bovine serum albumin in 800 ml of water. [NOTE - 2.00 g of human albumin may be substituted for 2.00 g of bovine serum albumin.] Adjust with hydrochloric acid to a pH of 8.4, and dilute with water to 1000 ml.

6. **Acetic Acid Solution(Stop Solution):** (Not provided in the kit):
   20% v/v Glacial Acetic Acid: 20 ml of Glacial Acetic Acid in 80 ml of Distilled Water.

7. **Standard and Test Concentration:** Recommended range of standard and Test concentration includes: 0.20 IU/ml, 0.10 IU/ml, 0.050 IU/ml, and 0.025 IU/ml.

For Example:

Preparation of Standard Concentrations

Standard Concentration 500 IU/ml (Main Stock) is to be diluted as per below table:

Standard Dilution

Sr No.	Concentration (IU/ml)	Stock (ul)	Diluent Buffer pH 7.4 (ul)	Total Volume (ul)
S1	50	50 ul of M.S	450	500
S2	1	20 ul of S1	980	1000
S3	0.20	60 ul of S2	240	300
S4	0.10	150 ul of S3	150	300
S5	0.050	150 ul of S4	150	300
S6	0.025	150 ul of S5	150	300

Test Dilution – Test Sample Main Stock is of concentration 500 IU/ml

Sr No.	Concentration (IU/ml)	Stock (ul)	Diluent Buffer pH 7.4 (ul)	Total Volume (ul)
T1	50	50 ul of M.S	450	500
T2	1	20 ul of T1	980	1000
T3	0.20	60 ul of T2	240	300
T4	0.10	150 ul of T3	150	300
T5	0.050	150 ul of T4	150	300
T6	0.025	150 ul of T5	150	300

Assay Protocol:
Add the reagents into the microwell as per following steps:

	Microwell
Anti-thrombin III	20 ul
Standard or Test Sample	20 ul
Mix but do not allow bubbles to form. Incubate at 37°C, for 1 minute.	
Bovine Factor Xa	50 ul
Mix and incubate at 37°C, for exactly 1 minute.	
Chromogenic Substrate	100 ul
Mix and incubate at 37°C, for 4 minutes.	
Acetic Acid	100 ul
Mix and measure the absorbance at 405 nm.	

Calculation of Results:

For each series, calculate the regression of the absorbance against log concentration of the sample solutions and the standard solutions. Calculate the potency of the enoxaparin in IU of Anti-Factor Xa activity/ml using statistical methods for parallel-line assays. The four independent log relative potency estimates are then combined to obtain the final geometric mean. Its confidence limits are calculated. Express the Anti-Factor Xa activity of the sample in mg.
Standard and Test Samples being serial diluted should pass the test for linearity and parallelism as the interpretation is done by extrapolating the data. We have used proprietary MS Excel software for the same based on DJ Finney algorithm.

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