

BIOPHEN Factor IXa (ACT. FIX)

Ref. 221812

Chromogenic assay for measuring Factor IXa activity.

In vitro research use only

HYPHEN BioMed

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INTENDED USE:

BIOPHEN Factor IXa (ACT. FIX) kit is a chromogenic assay for measuring activated Factor IX (FIXa) activity, using a chromogenic method, manual or automated.

APPLICATIONS:

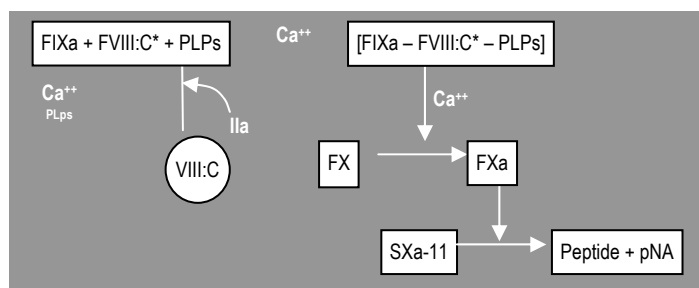
Assay of Factor IXa activity in any FIX therapeutic concentrate or purified milieu, where Factor IXa activity must be measured.

ASSAY PRINCIPLE:

In presence of Phospholipids (PLPs) and Calcium, activated FIX (FIXa), present in the tested sample, forms an enzymatic complex with thrombin activated factor VIII:C, also supplied in the assay at a constant concentration and in excess, that activates Factor X, present in the assay system, into Factor Xa.

This activity is directly related to the amount of Factor IXa, which is the limiting factor. Generated Factor Xa is then exactly measured by its specific activity on a Factor Xa chromogenic substrate (Sxa-11). Factor Xa cleaves the substrate and releases pNA. The amount of pNA generated is directly proportional to the Factor IXa activity.

Finally, there is a direct relationship between the amount of Factor IXa in the assayed sample and the Factor Xa activity generated, measured by the amount of pNA released, determined by colour development at 405nm.



Nota : FVIII:C: Thrombin activated FVIII:C*

REAGENTS:

R1: Reagent 1: Human Factor X and FVIII:C

Human Factor X, and FVIII:C, lyophilised in presence of a fibrin polymerisation inhibitor and stabilizers. 2 vials (to be reconstituted with 2.5 mL of distilled water).

R2: Reagent 2: "Activation" Reagent (Thrombin-Calcium-Phospholipids)

Human thrombin, calcium and synthetic phospholipids, lyophilised, in presence of stabilizers. 2 vials (to be reconstituted with 2.5 mL of distilled water).

R3: Reagent 3: Sxa-11 (Sequence: Suc-Ile-Glu-(γ-pip)Gly-Arg-pNA, HCl)

Chromogenic substrate, specific for Factor Xa (Sxa-11), lyophilised. 2 vials of Sxa-11 with a FXIa inhibitor (to be reconstituted with 2.5 mL of distilled water).

R4: Reagent 4: Tris-BSA Buffer

Tris-BSA Buffer, ready to use. Contains 1% BSA, Factor VIII:C stabilizers and sodium azide. (2 vials of 25 mL).

Cal: FIXa calibrator: (established against the International Standard)

Purified human Factor IXa, lyophilised. When restored with 1 ml of distilled water, a solution containing a concentration "C" (expressed in milli- International Units per ml (mIU/ml)) of human FIXa is obtained. This concentration (usually in the range 25-30 mIU/ml according to the lot), is accurately determined for each lot.

2 vials (to be reconstituted with 1 mL of distilled water).

The exact concentration of FIXa is indicated on the flyer provided in each kit. The calibration curve covers the range from 0 to about 30 mIU/ml. (1 mIU/ml is close to 1 ng/ml factor IXa)

Warning: - Human Factor X and Thrombin were prepared from human plasma, which was tested with registered methods and found negative for HIV antibodies, HBs Ag and HVC antibodies. Bovine Serum Albumin (BSA) was prepared from bovine plasma, which was tested for the absence of infectious agents, and collected from animals free from BSE. However, no assay may warrant the total absence of infectious agents. Any product of biological origin must then be handled with all the required cautions, as being potentially infectious.

- Sodium azide (0.9 g/l) may react with lead and copper plumbing to form highly explosive metal azides. Flush with large volumes of water when discarding into a sink.

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:

Reagents:

- Distilled water, preferentially sterile.
- Acetic Acid (20%) or Citric Acid (2%) (End point method).
- Quality Controls, at Low and High FIXa concentrations.
- Alternatively, reference material for Factor IXa (international or internal).

Material:

- Spectrophotometer, photometer or automates for chromogenic assays, with a wave-length set up at 405 nm.
- Stop watch.
- Calibrated pipettes.

STORAGE CONDITIONS:

BIOPHEN Factor IXa reagents must be stored at 2-8°C, in their original packaging box. They are then stable until the expiration date printed on the box.

PREPARATION AND STABILITY OF REAGENTS:

R1: Reagent 1: Human Factor X, FVIII:C and fibrin polymerization inhibitor

- Reconstitute each vial with exactly 2.5 mL of distilled water.
- Let to homogenize for 30 minutes at room temperature (18-25 °C).
- Shake gently before use.

Stability of reconstituted reagent R1, kept in its original vial:

- 72 hours at 2-8°C.
- 24 hours at room temperature (18-25 °C).
- 2 months at -20°C or below.

R2: Reagent 2: Thrombin, Phospholipids and Calcium

- Reconstitute each vial with 2.5 mL of distilled water. Shake thoroughly until complete dissolution of the content (vortex).
- Let to homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time.
- Homogenize the content before each use.

Stability of restored reagent R2, kept in its original vial:

- 72 hours at 2-8°C.
- 24 hours at room temperature (18-25 °C).
- 2 months at -20°C or below.

R3: Reagent 3: Factor Xa specific Chromogenic substrate (Sxa-11)

- Reconstitute each vial with 2.5 mL of distilled water. Shake thoroughly until complete dissolution of the content (vortex).
- Incubate at room temperature (18-25°C) for 30 minutes, while shaking the vial from time to time.
- Homogenize the content before each use.

Stability of restored substrate, kept in its original vial:

- 1 month at 2-8°C.
- 7 days at room temperature (18-25 °C).
- 2 months at -20°C or below.

R4: Reagent 4: Tris-BSA Buffer

Ready to use buffer. Shake before use.

Stability of the buffer, protected from any bacterial contamination:

- In its original vial, until the expiration date printed on the label, at 2-8°C.
- When open, 7 days at 2-8 °C

Cal: FIXa calibrator:

- Reconstitute each vial with 1 ml of distilled water. Shake thoroughly until complete dissolution of the content (vortex). A ready to use solution containing a concentration "C" (expressed in mIU/ml) of human FIXa is obtained. Incubate at room temperature (18-25°C) for 15 minutes, while shaking the vial from time to time. Homogenize the content before each use.

Stability of restored Calibrator, kept in its original vial:

- 72 hours at 2-8°C.
- 24 hours at room temperature (18-25 °C).
- Do not freeze.

Cautions:

- In order to improve stability, reagents must be closed with their original screw cap following each use (white caps for R1 and R2, yellow cap for R3, white cap for buffer R4, and blue cap for Cal).
- Reagents must be handled with care, in order to avoid any contamination during use.
- If the substrate becomes yellow, this indicates the presence of a contaminant. It must be rejected, and a new vial must be used.

Note:

- R1, R2, R3 and Cal vials are closed under vacuum. Remove carefully the stopper, in order to avoid any lost of powder when opening the vials.
- According to the automated method used, the reagents can be reconstituted with volumes different from those recommended. In any case, the established reactive ratios (respective reagent concentrations in the reactive milieu) between R1, R2 and R3 must be adhered to.
- Use only reagents from kits with the same lot number. Do not mix reagents from kits with different lots when running the assay. Reagents R1 and R2 are optimized for each lot of kits.
- The stability studies at 30°C show that the reagents can be shipped at room temperature without damage.

TESTED SPECIMEN:

Factor IXa in purified milieu or in FIX therapeutic concentrate.

TEST PROCEDURE:

BIOPHEN FIXa kit is designed for being used with automated kinetic methods but it can also be used for end point manual methods. Adaptations to the various automates are available upon request. The assay is performed at the controlled temperature of 37°C and the colour development is measured at 405 nm.

CALIBRATION:

Using the **FIXa Calibrator**, reconstituted with 1ml distilled water, and with a FIXa concentration "C" (usually in the range 25-30 mIU/ml, according to the lot used), provided in the kit, prepare the following calibrator dilutions:

Factor IXa concentration (mIU/ml)	C/2	C/4	C/8	C/20	C/40	0
Vol. of FIXa calibrator at C	0.5 ml	0.25 ml	0.125 ml	0.05ml	0.025 ml	0 ml
Vol. of R4 buffer	0.5 ml	0.75 ml	0.875 ml	0.95ml	0.975 ml	1 ml

Mix gently for a complete homogenisation.

The calibrator dilutions are stable for at least **4 hours** at room temperature (18-25°C).

Alternatively, the calibration curve can also be performed using a reference Factor IXa material (international standard or internal standard preparation).

Predilute the preparation (with the known FIXa content) **at least 1:2** with R4 dilution buffer, for obtaining the "C" mIU/ml Factor IXa concentration and prepare the calibration range as for a calibrator titrating "C" mIU/ml Factor IXa.

For Factor IXa concentrates, the tested specimen must be pre-diluted **at least 1:2** in R4 buffer, in order to obtain an expected Factor IXa concentration of about **C/2 mIU/ml or below** for the tested sample. If required, it is recommended to prepare a pre-dilution, in order to bring the expected Factor IXa concentration in the range **3 to 30 mIU/ml** with R4 buffer, and then dilute it **1:2** with R4 buffer for the assay. The factor IXa concentration is then expected in the range **1.5 to 15 mIU/ml** in the tested sample.

The measured concentration must then be multiplied by the dilution and the "pre-dilution" factors.

In order to get the full assay performances, the calibration curve must be prepared just before running the assay in order to avoid any FIXa degradation which could lead to erroneous results.

ASSAY PROTOCOL:

Manual Method:

Tested samples (in the expected range 3 to 30 mIU/ml) are assayed at least at the **1:2** dilution in Tris-BSA buffer (R4).

For therapeutic concentrates or solutions with Factor IXa concentrations different from those of FIX in plasma, predilute the tested specimen in order to have an expected Factor IXa concentration between **3 and 30 mIU/ml**, then dilute it **1:2** with R4 dilution buffer for the assay.

Reagents	Microplate	Test Tube
Ready to use calibrator, or diluted tested samples or Controls	50 µL	200 µL
R1 : Factor X-VIII:C	50 µL	200 µL
Mix and incubate for 2 min at 37°C, then introduce:		
R2 : Activation mixture	50 µL	200 µL
Mix and incubate for 3 min at 37°C, then introduce:		
R3: Sxa-11 Substrate preincubated at 37°C	50 µL	200 µL
Mix and incubate for 3 min at 37°C, exactly		
Stop the reaction by introducing:		
Citric Acid (20g/L), or 20 % Acetic Acid	50 µL	200 µL
Mix and measure the Absorbance at 405nm against the sample blank.		

The yellow colour obtained is stable for 2 hours.

The sample blank is obtained by mixing the reagents in the opposite order from that of the test i.e.: Citric Acid (20 g/L), Sxa-11 substrate, diluted sample, R1, R2.

Measure the Absorbance at 405 nm (A405). Subtract the sample blank from the **A405** obtained for the assay.

Kinetics mode:

The assay can be read using a kinetics mode. In this case the change in absorbance is recorded from 10 seconds to 100 seconds following the addition of substrate. There is then no need to subtract the sample blank, or to stop the reaction. The results are obtained using the change in absorbance ($\Delta A405$) for calibrators and tested specimen.

Automated methods:

Adaptations to the various analysers are available upon request. The assay is then performed kinetically. The reaction does not require to be stopped and sample blanks are automatically subtracted.

Note:

- If higher or lower reactive volumes than those indicated here above are required for the method used, the same respective proportions between reagent concentrations and volumes used, must be adhered to, in order to maintain the assay performances.
- Run a sample blank if the sample has a "colour" different from the usual one.

RESULTS:

- For the end-point method, use a **bilogarithmic** graph paper and plot on abscissae the Factor IXa concentration (mIU/ml) and on ordinates the corresponding absorbances (**A405**). The Factor IXa concentration in the diluted tested sample is directly obtained on the calibration curve. Results are expressed as mIU/ml of Factor IXa.
- When the kinetics mode is used, proceed the same way by plotting the **$\Delta A405$** values obtained, instead of A405.
- Using automated methods, the Factor IXa concentrations are directly calculated by the analyser, respectively to the calibration curve, and the sample dilution used.

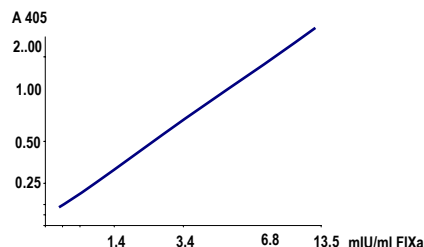
When the assay dilution is **1:2**, the Factor IXa concentration read on the calibration curve must be multiplied by **2**. When other predilutions are used, multiply the measured Factor IXa concentration by the complementary predilution factor in order to get the concentration in the tested specimen.

QUALITY CONTROL:

The control is performed with internal or commercially available controls, titrated for Factor IXa. Using quality controls, titrated for Factor IXa, allows validating the calibration curve, as well as the homogeneous reactivity from run to run and from series to series, when using a same lot of reagents.

EXAMPLE OF CALIBRATION CURVE:

The calibration curve below is an example only, using the end point manual method. Only the calibration curve generated for the series of assays performed must be used for calculating the Factor IXa concentrations.



TRACEABILITY TO THE REFERENCE MATERIAL:

The FIXa concentration of the FIXa Calibrator provided in the kit is exactly defined against an Internal Reference Standard, initially validated by reference to the 1st International Standard for activated Factor IX (FIXa), human (NIBSC) (code 97/562).

PERFORMANCE CHARACTERISTICS:

The detection threshold for the assay is evaluated on the calibration curve by measuring the "apparent" Factor IXa concentration, which corresponds to the mean A405 value obtained for a sample free of Factor IXa plus 3 Standard Deviations (SD). This detection threshold is of about **0.1 mIU/ml** (ie about 0.1 ng/ml).

BIOCHEMISTRY:

Factor IX (FIX) is a vitamin K dependent single chain glycoprotein of about 55 KDa, which participates in the middle phases of blood coagulation.

The normal Factor IX concentration in human plasma is of about 4 to 5 µg/ml.

When activated by factor XI, in the presence of calcium, FIX(a) forms an active complex with FVIII:C, in the presence of calcium and phospholipids, which converts FX into FXa.

REFERENCES:

1. Taran LD, "Factor IX of the blood coagulation system: a review", Biochemistry (Mosc.), 62(7):685-93, 1997.
2. Wagenvoort R, Hendrix H, Tran T, Hemker HC, "Development of a sensitive and rapid chromogenic FIX assay for clinical use", Haemostasis, 20(5): 276-88, 1990.
3. www.ncbi.nlm.nih.gov, OMIM, Haemophilia B, FIX deficiency, +306900, +134540, +134510, +134520.