BIOPHEN Factor IX (6)
Ref. 221806-RUO

Chromogenic assay for measuring
Factor IX activity in plasma or concentrates.

For Research Use Only.
Not for Use in Diagnostic Procedures

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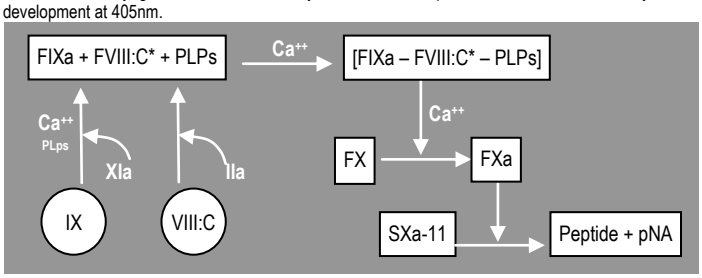
Last revision: 16/04/2015

INTENDED USE:

BIOPHEN Factor IX kit is a chromogenic assay for measuring Factor IX activity in human citrated plasma or in Factor IX concentrates, using a chromogenic method, manual or automated.
This kit is for research use only and should not be used for patient diagnosis or treatment

ASSAY PRINCIPLE:

In presence of thrombin, phospholipids and calcium, first Factor XIa, supplied in the assay at a constant concentration and in excess, activates FIX, present in the tested sample, into FIXa, which forms an enzymatic complex with thrombin activated factor VIII:C, also supplied in the assay at a constant concentration and in excess, phospholipids (PLPs) and Calcium, that activates Factor X, present in the assay system, into Factor Xa.
This activity is directly related to the amount of Factor IX, which is the limiting factor. Generated Factor Xa is then exactly measured by its specific activity on 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 IX in the assayed sample and the Factor Xa activity generated, measured by the amount of pNA released, determined by colour development at 405nm.



Note : FVIII:C: Thrombin activated FVIII:C*

REAGENTS:

R1: Reagent 1: Human Factor X and FVIII:C
Human Factor X, and FVIII:C, lyophilized in presence of a fibrin polymerization inhibitor and stabilizers.
2 vials (to be reconstituted with 6 mL of distilled water).

R2: Reagent 2: Activation Reagent (XIa-Thrombin-Calcium-phospholipids)
Factor XIa (human), at a constant and optimized concentration, containing human thrombin, calcium and synthetic phospholipids, lyophilized, in presence of stabilizers.
2 vials (to be reconstituted with 6 mL of distilled water).

R3: Reagent 3: SXa-11
Chromogenic substrate, specific for Factor Xa (SXa-11), lyophilized.
2 vials of SXa-11 with a FXIa inhibitor (to be reconstituted with 6 mL of distilled water).

R4: Reagent 4: Tris-BSA Buffer
Tris-BSA Buffer, ready to use. Contains 1% BSA, PEG, FVIII:C Stabilizer and sodium azide (0.9g/L). .
(4 vials of 25 mL).

Warning:
- Human Factor X, Factor XIa 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).
- Plasma Calibrator **BIOPHEN Plasma Calibrator Ref 222101** or equivalent
- Normal or Abnormal Quality Control Plasmas, titrated in FIX, **BIOPHEN Normal Control Plasma Ref 223201, and BIOPHEN Abnormal Control Plasma Ref 223301** or equivalent.
- Reference material for Factor IX concentrates (international or internal).

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

STORAGE CONDITIONS:

BIOPHEN Factor IX 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:

Note: Reconstitution volumes can vary according to the analyzer used. Refer to each specific instrument adaptation.

R1: Reagent 1: Human Factor X, FVIII:C and fibrin polymerization inhibitor
- Reconstitute each vial with exactly **6 mL** of distilled water; homogenize well.
- Let homogenize for 30 minutes at room temperature (18-25 °C).
- Shake gently before use.

Stability of reconstituted reagent R1, kept in its original vial:
- 24 hours at 2-8°C.
- 8 hours at room temperature (18-25 °C).
- 2 months at -20°C or below.

R2: Reagent 2: Factor XIa, with thrombin, phospholipids and Calcium
- Reconstitute each vial with **6 mL** of distilled water. Shake thoroughly until complete dissolution of the content.
- Let homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time.
- Homogenize the contents before each use.

Stability of restored reagent R2, kept in its original vial:
- 24 hours at 2-8°C.
- 8 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 **6 mL** of distilled water. Shake thoroughly. Let to homogenize for 30 minutes at room temperature (18-25 °C), while shaking the vial from time to time, until **complete dissolution** of the content. Check the absence of any solid at the bottom of the vial.

Warning: In all cases, before use, check the absence of solids at the bottom of the vial, which confirms that dissolution is complete. If necessary, incubate for 1 hour at RT or better at 37°C, while shaking from time to time. If required, then additionally incubate overnight at RT.

Stabilize at room temperature and homogenize the contents 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

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, and white cap for buffer R4).
- 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 and R3 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, R2 and R3 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:

Human citrated plasma or Factor IX concentrate.

PREPARATION OF PLASMA:

- Sample collection :

Blood (9 volumes) must be collected on 0.109 M citrate anticoagulant (1 volume), through a net venipuncture with great care, in order to avoid activation.

Centrifugation :

The centrifugation step is important and is intended to separate the plasma from the platelets. This must be performed quickly after blood collection using citrate tubes or CTAD (Citrate, Theophylline, Adenosine and Dipyridamole) tubes.

Use a validated method established by your laboratory to obtain platelet poor plasma. For example, 15 minutes at 2000g at room temperature (18-25°C). Use nonactivating plastic tubes and pipettes for handling and storage.

- Plasma Storage :
  - 8 hours at room temperature (18-25°C)
  - 24 hours at 2-8°C
  - 2 weeks at -20°C and up to 6 months at -70°C

Refer to NCCLS/CLSI document H21-A5 for further instructions on specimen collection, handling and storage.

TEST PROCEDURE:

BIOPHEN FIX 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 analyzers are available upon request. The assay is performed at the controlled temperature of **37°C** and the color development is measured at **405 nm**.

CALIBRATION:

High range (5 to 200%):

Calibration is performed with a commercially available plasma calibrator, titrated for FIX concentration, or with internal or international reference material for FIX concentrates.

When the calibration is performed with a commercially available plasma calibrator, with a known Factor IX concentration (**C**), the **1:100** dilution corresponds to the indicated Factor IX concentration, and the **1:50** to twice this concentration. Using a plasma calibrator with a Factor IX concentration of **C**, the **200%** FIX concentration is obtained (in the assay conditions) by using the following dilution factor:
50 x C:100.

BIOPHEN FIX kit can also be calibrated with a normal pooled citrated plasma (made with plasmas from at least 30 normal individuals, males or females, aged between 18 and 55 years, and free of any medication or disease), with the assigned value of **100 %** Factor IX. The assay includes a standard plasma dilution of **1:100**. By definition, this latter dilution of the pool represents the **100 %** Factor IX activity. The dynamic range is from **0 to 200 %** Factor IX. The **200 %** Factor IX activity is then the **1:50** dilution of the **plasma pool** (in Tris-BSA buffer (R4).

Prepare **2 ml** of the **1:50** dilution of the normal plasma pool, or of a (**50 x C:100)** dilution of the FIX reference standard. This corresponds to 200% FIX (noted **C1**); the calibration curve can then be obtained by preparing serial dilutions as follows:

Standard	C1	C2	C3	C4	C5
% FIX	200	100	50	25	5
Vol of FIX standard	1000µL of C1	500µL of C1	500µL of C2	500µL of C3	100µL of C4
Vol of Tris-BSA Buffer (R4)	0 µL	500µL	500µL	500µL	400µL

The calibration curve can also be performed using a reference Factor IX material (international standard or internal standard preparation).

Predilute the preparation (with the known FIX content) with **R4** dilution buffer, to exactly 1 unit/ml, then dilute it **1:50** with **R4** for obtaining the **200%** Factor IX concentration and prepare the calibration range as for a plasma pool titrating **100%** Factor IX.

For Factor IX concentrates, the tested specimen must be pre-diluted in **R4** in order to obtain an expected Factor IX concentration of about **1 IU/ml** for the tested sample. It is recommended to prepare a pre-dilution, in order to bring the expected Factor IX concentration in the range **0.2 - 2 IU/ml**, and then to dilute it **1:100** with **R4** for the assay. The factor IX concentration is then expected in the range **20 - 200%.**

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

Low range (0 to 20%):

Calibration is performed with a normal pooled citrated plasma with the assigned value of **100 %** Factor IX. It must be diluted **1:5** in a Factor IX deficient plasma (**Ref. DP050A/K-RUO**) in order to obtain a concentration of **20%** Factor IX (1 volume of normal pooled citrated plasma + 4 volumes of buffer or Factor IX deficient plasma). This normal citrated pooled plasma is then diluted **1:20** (in Tris-BSA buffer (R4)). By definition, this latter dilution of the pool represents the **20 %** Factor IX activity. The dynamic range is from **1 to 20 %** Factor IX.

Or calibration is performed with a commercially available plasma calibrator, with a known Factor IX concentration (**C**). Following reconstitution, the calibrator must be appropriately diluted in the Factor IX deficient plasma in order to obtain a Factor IX concentration of **20%** (the dilution factor is then **5 x C: 100)**. This calibrator is then diluted **1:20** (in Tris-BSA buffer (R4)). By definition, this latter dilution of the calibrator represents the **20%** Factor IX activity.

The calibration curve can then be prepared as follows:

% IX	20% IX Calibrator (µL)	Tris-BSA Buffer (R4) (µL)
1	25	475
2.5	65	455
5	125	375
10	250	250
20	500	0

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

ASSAY PROTOCOL:

Manual Method:

High range: Tested plasmas and controls are assayed at the **1:100** dilution in Tris-BSA buffer (R4).

For Factor IX concentrates or for biological fluids with Factor IX concentrations different from those of plasma, predilute the tested specimen in order to have an expected Factor IX concentration between **0.2** and **2** unit/ml, then dilute it **1:100** with **R4** dilution buffer for the assay.

Low range: Tested plasmas and controls are assayed at the **1:20** dilution in Tris-BSA buffer (R4).

Reagents	Microplate	Test Tube
Calibrators, or diluted tested plasmas, 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 **2** 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 gIL), SXa-11 substrate, diluted plasma, 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 (ΔA405) for calibrators and tested specimen.

Automated methods:

Adaptations to the various analyzers 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 in presence of highly lipemic, icteric or haemolysed plasmas, or if the plasmas has a "color" different from the usual one.

RESULTS:

- For the end-point method, use a **bilogarithmic** graph paper and plot on abscissae the Factor IX concentration **(%)** and on ordinates the corresponding absorbance (**A405**).
  The Factor IX concentration in the tested sample is directly obtained on the calibration curve. Results are expressed as % of Factor IX.
- When the kinetics mode is used, proceed the same way by plotting the **ΔA405** values obtained, instead of A405.
- Using automated methods, the Factor IX concentrations are directly calculated by the analyzer, respectively to the calibration curve, and the sample dilution used.
- The dynamic range is from **5 to 200 % for the high range** or **1 to 20% for the low range.**
- When the assay dilution is **1:100 (for the high range) or 1:20 (for the low range)**, the Factor IX concentration is directly read on the calibration curve. When different dilutions are used, the results must be multiplied by the dilution factor "**D**", divided by **100**, i.e. **D/100, for the high range,** and by **20**, i.e., **D/20, for the low range.**
- **The results obtained should be for research purposes only and not used for patient treatment or diagnosis.**

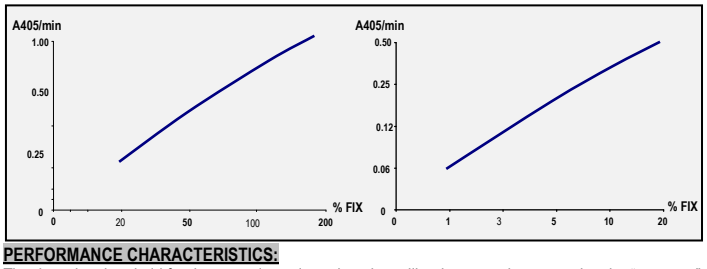
QUALITY CONTROL:

The control is performed with commercially available control plasmas, titrated for Factor IX.

Using quality control plasmas, titrated for Factor IX, 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. Various control plasmas are available, the FIX concentration should be confirmed by the laboratory in the usual working conditions: **BIOPHEN Normal Control Plasma (ref 223201)** and **BIOPHEN Abnormal Control Plasma (ref 223301).**

EXAMPLE OF CALIBRATION CURVE:

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



PERFORMANCE CHARACTERISTICS:

The detection threshold for the assay is evaluated on the calibration curve by measuring the "apparent" Factor IX concentration, which corresponds to the mean A405 value obtained for a sample free of Factor IX plus 3 Standard Deviations (SD). This detection threshold is of about **2% for the high range** and **about 0.5% for the low range.**

Example of reproducibility and repeatability values obtained for plasmas with various FIX:

samples	% IX	High range				Low range	
		Intra-assay % STA-R		Total reproducibility %		Total reproducibility %	
		N	CV %	N	CV %	N	CV %
sample 1	83	12	2.8	47	3.25	37	4.76
sample 2	32	12	3.9	47	5.85	37	8.84

LIMITATIONS OF THE PROCEDURE:

- No significant interference is observed for heparin concentrations up to 2 IU/ml, bilirubin concentrations <0.25 mg/ml, haemoglobin concentrations <5 mg/ml and triglycerides concentrations <5mg/ml.
- In order to get the optimal assay performances, the working instructions must be carefully observed. Each laboratory should verify performances in its exact working conditions.

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 XIa, 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.