**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 Xa, 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 Xa, 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 released is determined by color development at 405mm.

**REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED**

- **FXa + FVIII:C** + PLPs
- **Ca**
- **PLps**
- **Factor Xa**
- **Factor IXa**
- **Factor VIII:C**
- **Inhibitor Factor**

Finally, this 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 color development at 405mm.

**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:**

**R1:**
- Reagent 1: Human Factor X, FVIII:C and fibrin polymerization inhibitor
  - Reconstitute each vial with exactly 2.5 mL of distilled water; homogenize well.
  - Let homogenize for 30 minutes at room temperature (18-25°C).
  - Shake gently before use.

**R2:**
- Reagent 2: Factor Xa, with thrombin, phospholipids and Calcium
  - Reconstitute each vial with 2.5 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 content before each use.

**R3:**
- Reagent 3: Factor Xa specific Chromogenic substrate (SXa-11)
  - Reconstitute each vial with 2.5 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.
  - Stabilize at room temperature and homogenize the content before each use.

**R4:**
- Reagent 4: Tris-BSA Buffer
  - Ready to use buffer. Shake before use.
  - Stabilize 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

**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 EDTA (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.

**Note:** Reconstitution volumes can vary according to the analyzer used. Refer to each specific instrument application.
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. Applications 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 (0 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 FIX 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 diluted plasma (made from plasma 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 1:30 dilution of the plasma pool (in Tri-BSSA Buffer) (R4).

Prepare 2 ml of the 1:50 dilution of the normal plasma pool, or of a (5 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:

<table>
<thead>
<tr>
<th>Standard</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>% FIX</td>
<td>200</td>
<td>100</td>
<td>50</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>Vol of FIX standard</td>
<td>1000µL</td>
<td>500µL</td>
<td>500µL</td>
<td>500µL</td>
<td>100µL</td>
</tr>
<tr>
<td>Vol of Tri-BSSA Buffer (R4)</td>
<td>0 µL</td>
<td>500µL</td>
<td>500µL</td>
<td>500µL</td>
<td>400µL</td>
</tr>
</tbody>
</table>

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 nM/ml for the test specimen. It is recommended to prepare a pre-dilution, in order to bring the expected Factor IX concentration in the range 0.2 - 2 nM/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 diluted plasma with the assigned value of 100 % Factor IX. It must be diluted 1:5 in a Factor IX deficient plasma (Ref. DP050AXK) in order to obtain a concentration of 20% FIX (1 volume of normal pooled diluted plasma + 4 volumes of buffer or Factor IX deficient plasma). This normal diluted pooled plasma is then diluted 1:20 (in Tri-BSSA 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 Tri-BSSA 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:

<table>
<thead>
<tr>
<th>% IX</th>
<th>20% IX</th>
<th>Fix (µL)</th>
<th>Tris-BSSA Buffer (R4) (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20% IX</td>
<td>Fix (µL)</td>
<td>Tris-BSSA Buffer (R4) (µL)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25</td>
<td>475</td>
</tr>
<tr>
<td>2.5</td>
<td>65</td>
<td>455</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>125</td>
<td>375</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>250</td>
<td>250</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>500</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

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 Tri-BSSA buffer (R4).

For concentrates or for biological fluids with Factor IX concentrations different from those of plasma, pre-dilute 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 Tri-BSSA Buffer (R4).

The yellow color 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.: Clinic Acid (20µL), Slaa-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 mode:**

Applications for various analyzers are available upon request. The assay is then performed kinetically. The reaction does not need to be stopped and sample blanks are automatically subtracted.

**Results:**

For the end-point method, use a bilogarithmic graph paper and plot on abscissa the Factor IX concentration (%) and on ordinates the corresponding absorbances (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 A405 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 223301) 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:

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Microplate</th>
<th>Test Tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibrators, or diluted tested plasma, or Controls</td>
<td>50 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>R1 (= Factor VIII:C)</td>
<td>50 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>Mix and incubate for 2 min at 37°C, then introduce:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R2 (= Activation mixture)</td>
<td>50 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>Mix and incubate for 3 min at 37°C, then introduce:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R3 (= Slaa-11 Substrate preincubated at 37°C)</td>
<td>50 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>Mix and incubate for 2 min at 37°C, exactly</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Stop the reaction by introducing:

Clinik acid (20µL) or 20 % Acetic Acid 50 µL 200 µL

The performance characteristics are expressed as:

- Lowest detection threshold for FVIII:C, VI, IX, X: 0.5%.
- Lowest detection threshold for FIX: 0.25%.
- The CV for intra-assay % of total reproducibility % for FIX is 4.16.

**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.
- 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.
- 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.

**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 about 4 to 5 µg/ml. When activated by factor Xa, 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:**


D750-02/BI/1802-RUO