BIOPHEN Factor IX
Ref. 221802

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

In vitro research use only

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.

CLINICAL APPLICATIONS:
Assay of Factor IX activity in any biological fluid or therapeutic concentrate, where Factor IX activity must be measured.

ASSAY PRINCIPLES:
In presence of thrombin, phospholipids and calcium, first Factor Xla, 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 Xa 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 405 nm.

Note: FIXa: Thrombin activated FXa.

REAGENTS:

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

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

R2: Reaction Activation Reagent (Xia-Thrombin-Calcium-phospholipids)

Factiva (human), at a constant and optimised concentration, containing human thrombin, calcium and synthetic phospholipids, lyophilised, in presence of stabilizers. 2 vials (to be reconstituted with 2.5 mL of distilled water).

R3: SXa-11 

Chromogenic substrate, specific for Factor Xa (SXa-11), lyophilised. 2 vials of SXa-11 with a FXa 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).

Warning:
- Human Factor X, Factor Xla and Thrombin were prepared from human plasma, which was tested with registered methods and found negative for HIV antibodies, HbsAg and HCV 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 (ex: BIOPHEN Plasma Calibrator Ref 222101).
- Normal or Abnormal Quality Control Plasmas (ex: BIOPHEN Normal Control Plasma Ref 223201, and BIOPHEN Abnormal Control Plasma Ref 223301).
- Reference factor for Factor IX concentrates (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 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 automated use. Refer to each specific instrument adaptation.

R1:
- Reagent 1: Human Factor X, FXII:C and fibrin polymerization inhibitor
  - Reconstitute each vial with exactly 2.5 mL of distilled water; homogenize well.
  - 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:
- 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 Xla, with 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:
- 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 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 (vortex), until complete dissolution of the content. Check the absence of any solid at the bottom of the vial.

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

Caution:
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 loss 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 mixtures) 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 stored at room temperature without damage.

TESTED SPECIMEN:
Human citrated plasma or Factor IX therapeutic concentrate.

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 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:
High range (5 to 200%):

Calibration is performed with a normal pooled citrated plasma (made with plasmas from at least 30 normal individuals, males or females, aged between 16 and 55 years, and free of any medication or disease) to have 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 Tris-BSA Buffer (R4). BIOPHENT FIX kit can also be calibrated with a commercially available plasma calibrator, titrated for FIX concentration, or with internal or international reference material for FIX concentrates.

If 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 1:30 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 × C : 100.

Prepare 2 ml of the 1:30 dilution of the normal plasma pool, or of a (50 x C : 100) dilution of the FIX recombinant standard (in order to correspond to 200% FIX (titled C)), the calibration curve can then be obtained by preparing serial dilutions as follows:

<table>
<thead>
<tr>
<th>% FIX</th>
<th>Standard</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>200</td>
<td>100</td>
<td>50</td>
<td>25</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

Vol of Vol of FIX standard

1000µL 500µL 500µL 500µL 100µL

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).

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

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 µ/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 µ/mL, and then to dilute 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. DP0506/40K) 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 (R4j). By definition, this latter dilution of the pool represents the 20 % Factor IX activity. The dynamic range is from 1 to 20 % Factor IX activity.

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 (R4j)). 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 Calibrator (µL)</th>
<th>Tris-BSA Buffer (R4) (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25 µL</td>
<td>575 µL</td>
</tr>
<tr>
<td>2.5</td>
<td>66 µL</td>
<td>455 µL</td>
</tr>
<tr>
<td>5</td>
<td>125 µL</td>
<td>375 µL</td>
</tr>
<tr>
<td>10</td>
<td>250 µL</td>
<td>250 µL</td>
</tr>
<tr>
<td>20</td>
<td>500 µL</td>
<td>0 µL</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 Tris-BSA buffer (R4). For therapeutic 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 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).

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 0 nanoseconds 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 analyser are available upon request. The assay is then performed kinetically. The reaction does not require 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 ΔA405 values obtained, instead of A405.

Using automated methods, the Factor IX concentrations are directly calculated by the analyser, 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.

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: BIOPHENT Normal Control Plasma (ref 223201) and BIOPHENT 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.

ASSAY PROTOCOL:

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.

EXPECTED VALUES:

The normal Factor IX range is from 70 to 130 %. Factor IX (also called antihemophilic factor) deficiency (<25%) results in haemophilia B (congenital bleeding disorder). Low plasma activity is observed in patients under antivitamin K therapy, or in case of hepatic disease, diathesis, DIC. High levels of FIX could be an indicator of an increased risk for venous thrombosis.

BIOPHENT FIX (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 Xla, in the presence of calcium, FIXa forms an active complex with FVIII:C, in the presence of calcium and phospholipids, which converts FXa into FXa.

REFERENCES:


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