BIOPHEN FVIII:C (6)
Ref. 221406-ROU
Chromogenic assay for measuring Factor VIII:C in
plasma, or in concentrates.
FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

INTENDED USE:
BIOPHEN FVIII:C (6) kit is a chromogenic assay for measuring the Factor VIII:C activity in human plasma or in Factor VIII:C 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:
When activated by thrombin, Factor VIII forms an enzymatic complex with Factor IXa, phospholipids and calcium, which activates Factor X to Factor Xa. BIOPHEN Factor VIII:C is a chromogenic assay for testing the co-factor activity of Factor VIII:C. In a constant concentration and in excess, to Factor Xa. This activity is directly related to the amount of Factor VIII:C, which is the limiting factor in presence of a constant and in excess amount of Factor IXa. Generated Factor Xa is then measured by its activity on a specific 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 VIII:C in the assayed sample and the Factor Xa activity generated, measured by the amount of pNA released, determined by color development at 405nm.

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

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:
- Distilled water, preferably sterile.
- Acetic Acid (20%) or Citric Acid (2%) (End point method).
- Plasma Calibrator (BIOPHEN Plasma Calibrator Ref. 22101).
- Normal or Abnormal Control Plasmas (BIOPHEN Normal Control Plasma Ref 223201, and BIOPHEN Abnormal Control Plasma Ref 223301).
- Spectrophotometer, photometer or analyzers for chromogenic assays, with a wave-length set up at 405 nm.
- Calibrated pipettes.

Storage Conditions:
BIOPHEN FVIII:C (6) 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
- Human Factor X, lyophilized in the presence of a fibrin polymerization inhibitor. 2 vials containing Factor X (to be reconstituted with 6 mL of distilled water).
R2: Reagent 2: Activation Reagent
- Factor IXa (human), at a constant and optimized concentration, containing human thrombin, calcium and synthetic phospholipids, lyophilized. 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 containing 36 mg of Sxa-11 with a thrombin inhibitor (to be reconstituted with 9 mL of distilled water).
R4: Reagent 4+: Tris-BSA Buffer
- Tris-BSA Buffer, ready to use. Contains 1% BSA, PEG, FVII:C Stabilizer and sodium azide (0.9g/L). (4 vials of 25 mL).

Warning:
- Human Factor X, Factor IXa and Thrombin were prepared from human plasma, which was tested with registered methods and found negative for HIV antibodies, HBs Ag 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 biologic origin must then be handled with all the required caution, as being potentially infectious.
- Sodium azide (0.9 g/L) may react with lead and copper plumbing to form highly explosive metal azide. Flush with distilled water. Always perform the sampling through a needle venipuncture, avoiding 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.

Caution:
- In order to improve stability, reagents must be closed with their original screw cap followed each use (green cap for factor X (R1), red cap for Factor IXa with thrombin, calcium and phospholipids (R2), yellow cap for Sxa-11 (R3), and white cap for buffer (R4)).
- Reagents must be handled with care, in order to avoid any contamination during use.
- Do not mix 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.

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.

REAGENTS AND MATERIAL REQUIRED BUT NOT PROVIDED:
- Biophen FVI:C

PREPARATION OF PLASMA (SPECIMEN COLLECTION):
Blood (9 volumes) must be collected on 6.109 M saline anticoagulant (1 volume), with great care, in a silicon glass or a plastic tube. Sampling must be performed through a needle venipuncture, avoiding any blood activation.
- Within 2 hours, blood must be centrifuged at 3,000 g for 20 min at 18°C or below, and plasma decanted into a plastic container, using a plastic pipette.
- Storage of plasma:
  - Up to 1 hour at Room Temperature (18-25°C).
  - Up to 8 hours at 2-8°C, or in a melting ice bath.
  - Up to 1 month frozen at −20°C or below
Refer to NCCLS document H21-A2 for further instructions on specimen collection, handling and storage.

TEST PROCEDURE:
BIOPHEN FVII:C (6) kit is designed for use with automated kinetic methods but it can also be used for end point manual methods. Adaptations for 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%):
BIOPHEN FVIII:C kit can be calibrated with the BIOPHEN Plasma Calibrator (ref 222101). Calibration is performed with a normal pooled citrated plasma, with a known Factor VIII:C concentration (C). The 1:40 dilution corresponds to the indicated Factor VIII:C concentration, and the 1:20 to twice this concentration. Using a plasma calibrator with a Factor VIII:C concentration of C, the 200 % Factor VIII:C concentration is obtained (in the assay conditions) by using the following dilution factor: 20 x C:100. The calibration curve can then be prepared as follows:

<table>
<thead>
<tr>
<th>% FVIII:C</th>
<th>200% FVIII:C Calibrator (µL)</th>
<th>Tris-BSA Buffer (R4+)(µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>50</td>
<td>125</td>
<td>375</td>
</tr>
<tr>
<td>100</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>200</td>
<td>500</td>
<td>0</td>
</tr>
</tbody>
</table>

For Factor VIII:C concentrates, the test sample must be pre-diluted in R4+ in order to have an expected Factor VIII:C concentration below 2 IU/ml for the test sample. It is recommended to prepare a pre-dilution, in order to bring the expected Factor VIII:C concentration in the range 0.2 - 2 IU/ml, and then to dilute in 1:40 for the assay. The Factor VIII:C concentration is then expected in the range 20 - 200%.

Low range (0 to 25%):
Calibration is performed with a normal pooled citrated plasma with the assigned value of 100% Factor VIII:C. It must be diluted 1:4 in a Factor VIII:C deficient plasma (Ref. DPA0A-K-RUO) in order to obtain a concentration of 25% Factor VIII:C (1 volume of normal pooled citrated plasma + 3 volumes of Factor VIII:C deficient plasma). This normal pooled plasma is then diluted 1:10 in (Tris-BSA buffer (R4+)). By definition, this latter dilution of the pool represents the 25% Factor VIII:C activity. The dynamic range is from 0 to 25 % Factor VIII:C.

For calibration is performed with a commercially available plasma calibrator, with a known Factor VIII:C concentration (C). The calibration curve can then be prepared as follows:

<table>
<thead>
<tr>
<th>% FVIII:C</th>
<th>25% FVIII:C Calibrator (µL)</th>
<th>Tris-BSA Buffer (R4+)(µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>6.25</td>
<td>125</td>
<td>375</td>
</tr>
<tr>
<td>12.5</td>
<td>250</td>
<td>250</td>
</tr>
<tr>
<td>25</td>
<td>500</td>
<td>0</td>
</tr>
</tbody>
</table>

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

ASSAY PROTOCOL:

Manual Method:
High range: Tested plasmas and controls are assayed at the 1:40 dilution in Tris-BSA buffer (R4+).

For Factor VIII:C concentrates with Factor VIII:C concentrations different from that of plasma, a dilution in Tris-BSA buffer is done to get a final Factor VIII:C concentration in the tested dilution in the range 0.005 to 0.050 IU/ml (i.e., 20 to 200 % Factor VIII:C, using this protocol).

Low range: Tested plasmas and controls are assayed at the 1:10 dilution in Tris-BSA buffer (R4+).

In a microplate well, or in a plastic (high range)

Calibrator, or diluted tested plasmas, or Controls

Mix and incubate for 5 min at 37°C, then introduce:

R3: SKa-11 Substrate preincubated at 37°C
Mix and incubate for 5 min at 37°C, exactly
Stop the reaction by introducing:

Citrin Acid (20g/L), or 20 % Acetic Acid
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., Citrin Acid (20 µL), SKa-11 substrate, diluted plasma, Factor X, Factor IXa mixture. Measure the Absorbance at 405 nm (A405). Subtract the sample blank from the A405 obtained for the assay.

Automated methods:
Applications for 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.

NB:
- 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 performance.
- Run a sample blank in presence of highly Ipronic, icteric or haemolysed plasmas, or if the plasma has a "colour" different from the usual one.

RESULTS:

- For the end-point method, use a bi-logarithmic (for the high range) or a linear (for the low range) graph paper and plot on abscissae the Factor VIII:C concentration (%) and on ordinates the corresponding absorbance (A405).
- The Factor VIII:C concentration in the tested sample is directly obtained on the calibration curve. Results are expressed as % of Factor VIII:C.
- Using automated methods, the Factor VIII:C 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, and 1 to 25 % for the low range. When the assay dilution is 1:40 (for the high range) or 1:10 (for the low range), the Factor VIII:C 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 40, i.e., D40, for the high range, and by 10, i.e., D10, for the low range.

The results obtained should be for research purposes only and not used for patient diagnosis or treatment.

QUALITY CONTROL:
The control is performed with commercially available control plasmas. Using quality control plasmas, titrated for Factor VIII:C, allows validating the calibration curve, as well as the homogeneity readvity from run to run and from series to series, when using a same lot of reagents. Various control plasmas are available:
BIOPHEN Normal Control Plasma: (ref 223301). BIOPHEN Abnormal Control Plasma: (ref 223301).

EXAMPLE OF CALIBRATION CURVE:
The high and low range calibration curves below are an example only, on STA. Only the calibration curve generated for the series of assays performed must be used for calculating the Factor VIII:C concentrations.

PERFORMANCE CHARACTERISTICS:

- The detection threshold for the assay is evaluated on the calibration curve by measuring the "apparent" Factor VIII:C concentration, which corresponds to the mean A405 value obtained for a sample free of Factor VIII:C plus 3 Standard Deviations (SD). This detection threshold is of about 10% (high range) and 2% (low range) for the BIOPHEN Factor VIII:C kit.

- Example of reproductibility values obtained for plasmas with various FVIII:C concentrations (STA)

- BIOCHEMISTRY:
Factor VIII:C is a plasma protein of about 230,000 daltons (230 KD). The synthesis site is still discussed, but it is thought to implicate endothelial cells. It is present in plasma at very low concentrations (<100 ng/ml). In blood, Factor VIII:C is stabilized by its binding to von Willebrand Factor (vWF), a multimeric glycoprotein (MW from 1 to 2 x10^6) which dramatically prolongs its half-life in blood circulation. In the absence of vWF, Factor VIII:C activity is rapidly cleared from blood.

REFERENCES:

D750-02/B1/1406-RUO