INTENDED USE:
The BIOPHEN™ FVIII:C kit is a chromogenic method for in vitro quantitative determination of Factor VIII activity (FVIII:C) on citrated human plasma or therapeutic concentrates, using a manual or automated amyloidylic method.

SUMMARY AND EXPLANATION:
Technical: Factor VIII is an approximately 280 kDa plasma protein. It is present in plasma at very low concentrations (100-200 ng/mL). In blood, FVIII is stabilized by its binding to von Willebrand Factor (vWF), which dramatically prolongs its half-life in blood circulation. In the absence of vWF, FVIII activity is rapidly cleared from blood.

Clinical: Factor VIII (or antihemophilic factor A) deficiency leads to the hemophilia A disease, a congenital coagulation disorder. Factor VIII levels are reduced in von Willebrand’s disease (vWD) or in case of Disseminated Intravascular Coagulation (DIC) or acquired FVIII inhibitor. Elevated concentrations of FVIII are observed in inflammatory or hepatic diseases and may be suggestive of an increased risk of venous thrombosis. The FVIII concentration is increased during pregnancy.

PRINCIPLE:
The BIOPHEN™ Factor VIII:C method involves the chromogenic assay of FVIII:C cofactor. In the presence of phospholipids (PLPs) and calcium, FVIII:C activated by thrombin, forms an enzyme complex with Factor Xa, which activates Factor X. The resulting Factor Xa hydrolyzes the chromogenic substrate, leading to the release of paranitroaniline (pNA). The amount of pNA released (measured by absorbance at 405 nm) is directly proportional to the concentration of FVIII:C in the specimen (Factor Xa, Thrombin and Factor X are in constant excess amount).

[Chemical Equation]

**REAGENTS:**

- **Human Factor Xa:** lyophilized. Contains a fibrin polymerization inhibitor, BSA and stabilizing agents.
- **Activation Reagent (IXa – Thrombin – Calcium – Phospholipids):** lyophilized. Contains human Factor IXa, at a constant and optimized concentration, human thrombin, calcium, synthetic phospholipids, stabilizing agents and BSA.
- **SXa-11:** chromogenic substrate, specific to Factor Xa (SXa-11), lyophilized. Contains a thrombin inhibitor.
- **Tris-BSA Buffer:** liquid. Contains 1% BSA, PEG, FVIII:C stabilizing agents and small amount of sodium azide (0.9 g/L) as preservative.

**WARNING AND PRECAUTIONS:**

- Some reagents provided in these kits contain materials of human and animal origin. Whenever human plasma is required for the preparation of these reagents, approved materials should be used to test the plasma for the antibodies to HIV 1, HIV 2 and HCV, and for hepatitis B surface antigen, and results are found to be negative. However, no test method can offer complete assurance that infectious agents are absent. Therefore, users of these reagents should be aware of the potential risk of infectious diseases.
- In contact with lead or copper pipes, sodium azide can generate explosive compounds.
- Waste should be disposed of in accordance with applicable local regulations.
- Use only the reagents from the same batch of kits.
- Aging studies show that the reagents can be shipped at room temperature without degradation.
- This device of diagnostic use is intended for professional use in the laboratory.

**REAGENT PREPARATION:**

- Gently remove the freeze-drying stopper, to avoid any product loss when opening the vial.

**REFERENCE INFORMATION:**

- **Reagents:**
  - **Plasma Calibrator:**
    - **Plasma Dilution:** 1:40
    - **Plasma Preparation:**
      - 100% FVIII:C level. The calibration curve ranges from 0 to 200%.
  - **Reference Materials:**
    - **Specific calibrators and controls with known titer, such as:**

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIOPHEN® Plasma Calibrator</td>
<td>222101</td>
</tr>
<tr>
<td>BIOPHEN® Normal Control Plasma</td>
<td>222301</td>
</tr>
<tr>
<td>BIOPHEN® Abnormal Control Plasma</td>
<td>223301</td>
</tr>
</tbody>
</table>

- **For low-range calibration, dilute the calibrator in FVIII:C deficient plasma (DP040A/K).** Also refer to the specific application guide of the analyzer used.

**SPECIMEN COLLECTION AND PREPARATION:**

- The blood (9 volumes) should be carefully collected onto the trisodium citrate anticoagulant (1 volume) (0.109 M, 3.2%) by clean venipuncture. Discard the first tube.
- Specimens should be prepared and stored in accordance with applicable local guidelines (for the United States, see the CLSI H21-A5* guideline for further information concerning specimen collection, handling and storage).

For plasma storage, please refer to references 5, 10, 11.

**PROCEDURE:**

- The kit can be used for kinetics, automated or manual (endpoint) methods. Perform the test at 37°C and read color intensity at 405 nm.
- For an automated method, application guides are available on request. See specific application guide and specific precautions for each analyzer.

**ASAY METHOD:**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Reconstitute the contents of each vial with exactly :</td>
</tr>
<tr>
<td>2</td>
<td>Reconstitute the contents of each vial with exactly :</td>
</tr>
<tr>
<td>3</td>
<td>Reconstitute the contents of each vial with exactly :</td>
</tr>
</tbody>
</table>

Prepare 1 mL of the 1/20 normal plasma pool dilution, or a (20x/C/100) dilution of the FVIII:C titrated calibrator plasma (i.e. C1). This solution has a FVIII:C titer of 200%. Prepare the following calibration curve by serial dilution of the buffer, as described in the following table:
The calibration curve can be established from a FVIII:C titrated reference material (international standard or internal standard). Pre-dilute this material in [R4] buffer to obtain a 1 IU/mL solution, then dilute 1:20 in R4 to obtain a solution with a 200% (4 IU/mL) FVIII:C factor. Use this solution to establish a calibration curve in [R4] buffer as previously explained.

Low range (0 to 25%): Calibration can be performed using a pool of citrated normal plasmas, or a commercial calibration plasma with a known concentration of FVIII:C, i.e. Control. Dilute this plasma in FVIII:C-deficient plasma (DPM45/5A) to achieve a 25% concentration (the dilution factor in deficient plasma is of 4 for the normal pool and of 4x/C100 for a calibrator with a concentration C).

The assay method includes a 1:10 plasma dilution. The calibration curve ranges from 0 to 25% FVIII:C. The 1:10 dilution in [R4] buffer represents 25% FVIII:C.

Using this solution, establish the following calibration curve in [R4] buffer:

<table>
<thead>
<tr>
<th>FVIII:C (%)</th>
<th>0</th>
<th>5</th>
<th>10</th>
<th>15</th>
<th>20</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of 25% FVIII:C Calibrator</td>
<td>0 µL</td>
<td>125 µL</td>
<td>250 µL</td>
<td>500 µL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume of R4 buffer</td>
<td>500 µL</td>
<td>375 µL</td>
<td>250 µL</td>
<td>0 µL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Prepare the calibration curve immediately before use to avoid any FVIII:C degradation.

2. Dilute the specimens in [R4] buffer, as described in the table below:

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Reference</th>
<th>Range</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>223301/223301</td>
<td>Low (after 1:10 pre-dilution in FVIII:C-deficient)</td>
<td>1:10</td>
</tr>
<tr>
<td>Specimens</td>
<td>N.A.</td>
<td>High</td>
<td>1:40</td>
</tr>
<tr>
<td>Low</td>
<td>1:10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For FVIII:C therapeutic concentrates, pre-dilute the test specimen (high range) in [R4] aiming for a FVIII:C concentration of approximately 1 IU/mL. We recommend performing a pre-dilution, in order to adjust the theoretical FVIII:C concentration to between 0.2 and 2 IU/mL then dilute 1:40 in [R4] to perform the test. The expected FVIII:C concentration is thus of between 20 and 200%. (The measured concentration should then be multiplied by the "pre-dilution" factor).

Establish the calibration curve and test it with the quality controls. If stored at room temperature (18-25°C), test the diluted specimens quickly. The exact calibrator and control concentrations for each batch are indicated on the flyer provided with the kit.

3. Dispense the following to the wells of a microplate, or to a plastic tube incubated at 37°C:

<table>
<thead>
<tr>
<th>Specimen, control or diluent in [R4]</th>
<th>Microplate</th>
<th>Test tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1 Human Factor X Pre-incubated at 37°C</td>
<td>50 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>R2 Activation Reagent Pre-incubated at 37°C</td>
<td>50 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>Mix and incubate at 37°C for 5 minutes, then add the following:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[R3] 5x1-11 Pre-incubated at 37°C</td>
<td>50 µL</td>
<td>100 µL</td>
</tr>
</tbody>
</table>

Mix and incubate at 37°C for 5 minutes exactly:

<table>
<thead>
<tr>
<th>Concentration (IU/mL)</th>
<th>50</th>
<th>200</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mix and measure the optical density at 405nm against the corresponding blank.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

"Or acetic acid (20%). The yellow color is stable for 2 hours. The specimen blank is obtained by mixing the reagents in the reverse order to that of the test: Citric acid (2%), R3, R2, R1, dilute specimen. Measure the optical density at 405 nm. Subtract the measured blank value from the absorbance measured for the corresponding test.

Create a plasma blank if sample is icteric, lipemic, haemolyzed, or if its color differs from the standard plasmas.

Kinetic method:
The assay can be performed by the kinetic method by measuring the change in absorbance between 10 and 100 seconds after adding the substrate (i.e. aLA405). In this case, there is no need to subtract the specimen blank, or to stop the reaction.

If a reaction volume other than that specified above is required for the method used, the ratio of volumes must be strictly observed to guarantee assay performance. The user is responsible for validating any changes and their impact on all results.

CALIBRATION:
The BIOPHEN™ FVIII:C assay can be calibrated for the assay of FVIII:C in plasma or therapeutic concentrates. The plasma calibrator covering the calibration range is available from HYPHEN BioMed (see the REAGENTS AND MATERIALS REQUIRED BUT NOT PROVIDED paragraph) and can be used to establish the calibration curve.

- The calibration range is about 17 to 195% (high range) or about 0.7 to 26% (low range) on Sysmex CS-series.

The calibration curves shown below are given by way of example only. The calibration curve established for the assay series must be used.

QUALITY CONTROL:
The use of quality controls serves to validate method compliance, along with between-test assay homogeneity for a given batch of reagents.

Include the quality controls with each series, as per good laboratory practice, in order to validate the test. A new calibration curve should be established, preferably for each test series, and at least for each new reagent batch, or after analyzer maintenance, or when the measured quality control values fall outside the acceptance range for the method.

Each laboratory must define its acceptance ranges and verify the expected performance in its analytical system.

RESULTS:
- For the manual endpoint method, plot the calibration curve log-log (high range) or lin-lin (low range), with the OD 405 nm along the Y-axis and the FVIII:C concentration, expressed as %, along the X-axis. When employing the kinetic method, use AOD 405 instead of OD 405.
- The concentration of FVIII:C (%) in the test specimen is directly inferred from the calibration curve, when the standard dilution is used.
- If other dilutions are used, the level obtained should be multiplied by the additional dilution factor.

The results should be interpreted according to the patient's clinical and biological condition.

LIMITATIONS:
- To ensure optimum test performance and to meet the specifications, the technical instructions validated by HYPHEN BioMed should be followed carefully.
- Any reagent presenting an unusual appearance or showing signs of contamination must be rejected.
- Any suspicious samples or those showing signs of activation must be rejected.
- FVIII:C result may be apparently affected in patients treated with direct Xa inhibitors or with particular FVIII mutations.

EXPECTED VALUES:
The normal FVIII:C level for adult plasma is usually in the range of 50 to 150%. However, each laboratory has to determine its own normal range.

PERFORMANCES:
- The lower detector limit depends on the analytical system used (<2% in high range and <0.5% in low range on Sysmex CS-5100).
- The measuring range depends on the analytical system used, about 2.5 to 250% for the high range and about 0.25 to 30% for the low range on Sysmex CS-series.
- Performance studies were conducted internally on Sysmex CS-5100. Performance was assessed using laboratory controls over a 25-day period, 2 series per day and duplicate within each series for a control level. The following results were obtained:

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>Bilirubin (C/L)</th>
<th>Heparins (LMWH/UFH)</th>
<th>Intralipids</th>
<th>Apixaban</th>
<th>Dabigatran</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 mg/dL</td>
<td>60 mg/dL</td>
<td>2 IU/mL</td>
<td>600 mg/dL</td>
<td>50 ng/mL</td>
<td>50 ng/mL</td>
</tr>
</tbody>
</table>

INCLUDE THE SPECIFIC APPLICATION GUIDE OF THE ANALYZER USED.

REFERENCES:
5. Carro M, et al. Comparison of reagents for factor VIII concentration (ISCOMB/BIOPHEN® Factor VIII:C Calibrator) on Sysmex CS-5100: n = 86; r = 0,975 x 7,19 1 = 0,989.
8. CLSI Document H21-A5: "Collection, transport, and processing of blood specimens for testing plasma-based coagulation assays and molecular hemostasis assays; approved guideline". 2008

SYMBOLS:
- Symbols used and signs listed in the ISO 15223-1 standard, see Symbol definitions document.

R1 H2 H3 H4
- Rw: Causes skin irritation.
- H3: Causes serious eye irritation.
- H35: May cause respiratory irritation.