



BIOPHEN FVIII:C (6) Ref. 221406

Chromogenic assay for measuring Factor VIII:C in plasma, or in concentrates.

In vitro diagnostic use only

Not for Sale in the US

Last revision: 16/04/2015

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.

CLINICAL APPLICATIONS:

Diagnosis of congenital or acquired Factor VIII:C deficiencies (Haemophilia A).

Assay of Factor VIII:C activity, in citrated human plasma or in therapeutic concentrates, where Factor VIII:C activity must be measured.

Follow-up of Factor VIII:C recovery in treated patients.

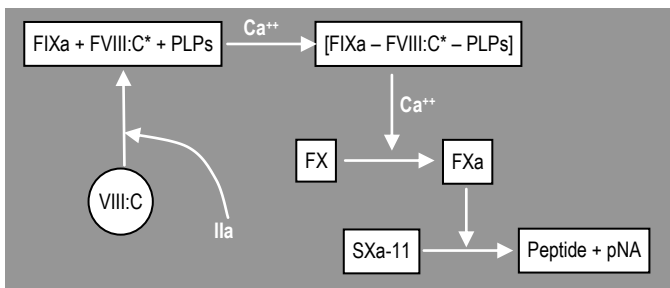
ASSAY PRINCIPLE:

When activated by thrombin, Factor VIII:C 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 cofactor activity of Factor VIII:C.

In presence of a constant amount of Factor IXa, Phospholipids (PLPs) and Calcium, thrombin activated Factor VIII:C forms an enzymatic complex, which activates Factor X, supplied in the assay at 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 exactly 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.



Nota : FVIII:C*: Thrombin activated FVIII:C

REAGENTS:

R1: Reagent 1: Human Factor X

Human Factor X, lyophilized in 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 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 IXa 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).
- Normal or Abnormal Control Plasmas (BIOPHEN Normal Control Plasma Ref 223201, and BIOPHEN Abnormal Control Plasma Ref 223301).

Material:

- Spectrophotometer, photometer or automates for chromogenic assays, with a wave-length set up at 405 nm.
- Stop watch.

- 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 and fibrin polymerization inhibitor

- Reconstitute each vial with exactly 6.0 mL of distilled water. Shake 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.

Stability of reconstituted human Factor X, kept in its original vial:

- 72 hours at 2-8°C.
- 24 hours at room temperature (18-25 °C).
- 2 months at -20°C or below.

R2: Reagent 2: Factor IXa, with thrombin, phospholipids and Calcium

- Reconstitute each vial with exactly 6.0 mL of distilled water. Shake 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.

Stability of restored reagent, kept in its original vial:

- 72 hours at 2-8°C.
- 24 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 exactly 6.0 mL of distilled water. Shake thoroughly. Let 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 content before each use.

Stability of restored substrate, kept in its original vial:

- 3 months 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 (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.
- 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 milieu) between Factor X, Factor IXa mixture and the Factor Xa substrate 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.

PREPARATION OF PLASMA (SPECIMEN COLLECTION):

Blood (9 volumes) must be collected on 0.109 M citrate anticoagulant (1 volume), with great care, in a silicon glass or a plastic tube. Sampling must be performed through a net 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 tube, 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 FVIII: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 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 (0 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 18 and 55 years, and free of any medication or disease), with the assigned value of 100 % Factor VIII:C. The assay includes a standard plasma dilution of 1:40. By definition, this latter dilution of the pool represents the 100 % Factor VIII:C activity. The dynamic range is from 0 to 200 % Factor VIII:C. The 200 % Factor VIII:C activity is then the 1:20 dilution of the plasma pool (in Tris-BSA buffer (R4+)).

BIOPHEN FVIII:C kit can be calibrated with the BIOPHEN Plasma Calibrator (ref 222101).

Or calibration is performed with a commercially available plasma calibrator, 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% FVIII: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:

% FVIII:C	200% VIII:C Calibrator (µL)	Tris-BSA Buffer (R4+) (µL)
0	0	500
50	125	375
100	250	250
200	500	0

For Factor VIII:C concentrates, the tested specimen must be pre-diluted in R4+ in order to have an expected Factor VIII:C concentration below 2 IU/ml for the tested 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 it 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. DP040A/K) 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 citrated 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.

Or calibration is performed with a commercially available plasma calibrator, with a known Factor VIII:C concentration (C). Following reconstitution, the calibrator must be appropriately diluted in the Factor VIII:C deficient plasma in order to obtain a Factor VIII:C concentration of 25% (the dilution factor is then 4 x C : 100). This calibrator is then diluted 1:10 (in Tris-BSA buffer (R4+)). By definition, this latter dilution of the calibrator represents the 25 % Factor VIII:C activity.

The calibration curve can then be prepared as follows:

% FVIII:C	25% VIII:C Calibrator (µL)	Tris-BSA Buffer (R4+) (µL)
0	0	500
6.25	125	375
12.5	250	250
25	500	0

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 therapeutic concentrates with Factor VIII:C concentrations different from that of plasma, dilute the sample in order 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 tube preincubated at 37°C, introduce:

Reagents	Microplate	Test Tube
Calibrators, or diluted tested plasmas, or Controls	50 µL	100 µL
R1 : Factor X preincubated at 37°C	50 µL	100 µL
R2 : Factor IXa mixture preincubated at 37 °C	50 µL	100 µL
Mix and incubate for 5 min at 37°C, then introduce:		
R3: SXa-11 Substrate preincubated at 37°C	50 µL	100 µL
Mix and incubate for 5 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 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.: Citric Acid (20 g/L), SXa-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:

Adaptations 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 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 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 analyser, 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. D/40, for the high range, and by 10, i.e. D/10, for the low range.

QUALITY CONTROL:

The control is performed with commercially available control plasmas..

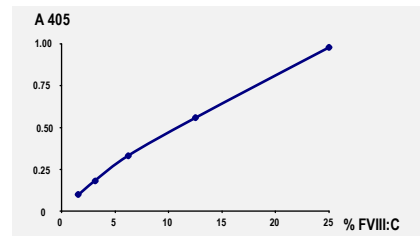
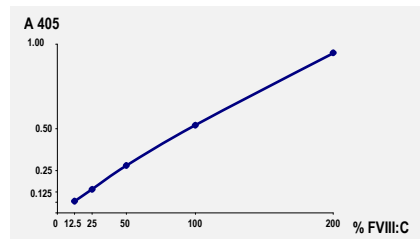
Using quality control plasmas, titrated for Factor VIII:C, allows validating the calibration curve, as well as the homogeneous reactivity from run to run and from series to series, when using a same lot of reagents. Various control plasmas are available:

BIOPHEN Normal Control Plasma: (ref 223201).

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 reproducibility values obtained for plasmas with various FVIII:C concentrations (STA) :

Samples	% VIII :C	Intra-assay CV %	N	Inter-assay CV %	N
Sample 1	76	2.8	7	6.1	7
Sample 2	58	4.2	7	4.8	7
Sample 3	46	2.8	7	3.4	7

EXPECTED VALUES:

The normal Factor VIII:C range is from 50 to 200 %.

Factor VIII:C is decreased in Haemophilia A or Von Willebrand's disease (vWd).

Elevated concentrations of Factor VIII:C are observed in inflammatory or hepatic diseases.

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 20 x10⁶ daltons) which dramatically prolongs its half-life in blood circulation. In the absence of vWF, Factor VIII:C activity is rapidly cleared from blood.

REFERENCES:

1. Wagenvoort RJ et al. Development of a simple chromogenic factor VIII assay for clinical use. *Haemostasis* Vol. 19, N°4, 196-204 (1989).
2. Mikaelsson M. et al. Influence of phospholipids on the assessment of factor VIII activity. *Haemophilia* Vol. 4, N°4, 646-50 (1998).
3. Lee CA. et al. Pharmacokinetics of recombinant factor VIII (recombinate) using one-stage clotting and chromogenic factor VIII assay. *Thromb. Haemost.* Vol. 82, N° 6, 1644-7 (1999).
4. Lundblad RL. et al. Issues with the assay of factor VIII activity in plasma and factor VIII concentrates. *Thromb. Haemost.* Vol 84, N° 6, 942-8 (2000).
5. Hubbard AR. et al. Potency estimation of recombinant factor VIII: effect of assay method and standard. *Br. J. Haematol* Vol. 113, N° 2, 533-6 (2001).
6. Raut S. et al. A collaborative study to establish the 6th International Standard for factor VIII concentrate. *Thromb. Haemost.* Vol. 85, N° 6, 1071-8 (2001).
7. Barrowcliffe TW. et al. Coagulation and chromogenic assays of factor VIII activity: general aspects, standardization, and recommendations. *Semin. Thromb. Hemost.* Vol 28, N° 3, 247-56 (2002).
8. Kirschbaum N. et al. Calibration of the Ph. Eur. BRP Batch 3/Mega 2 (US/FDA) standard for human coagulation factor VIII concentrate for use in the potency assay. *Pharmaceutical Spec Issue Biol Vol 1*, 31-64 (2002).
9. Mikaelsson M. et al. Assaying the circulating factor VIII activity in haemophilia A patients treated with recombinant factor VIII products. *Semin. Thromb. Hemost.* Vol 28, N° 3, 257-64 (2002).