ROX FACTOR VIII – 80 00 70

For Research Use Only – 2 x 100 tests

ENGLISH – Insert Revision September 20, 2018

1 INTENDED USE

Rox Factor VIII is a chromogenic kit for the determination of Factor VIII (FVIII) activity in human plasma and FVIII containing concentrates. This kit is for in vitro research use only and should not be used for patient diagnosis or treatment.

2 BIOCHEMISTRY

FVIII is a plasma protein of about 230 000 Daltons (230kD), which serves as a cofactor to Factor IXa in its activation of Factor X to Factor Xa. Deficiency of FVIII causes the severe bleeding disorder Hemophilia A. FVIII is stabilized by its binding to von Willebrand Factor (vWF), a multimeric glycoprotein which prolongs the half-life of FVIII in blood circulation.

3 MEASUREMENT PRINCIPLE

In the presence of Ca^{2+} and phospholipids, Factor X is activated to Factor Xa by Factor IXa. This reaction is greatly stimulated by FVIII after activation to FVIIIa by thrombin. By using optimal concentrations of Ca^{2+}, phospholipids and an excess of Factor IXa, Factor X and thrombin, the rate of activation of Factor X is directly related to the amount of FVIII in the sample. Factor Xa hydrolyses the chromogenic Factor Xa substrate, Z-D-Arg-Gly-Arg-pNA, thus liberating the chromophoric group pNA. The colour is read photometrically at 405 nm and the generated FXa and thus the intensity of colour is proportional to the FVIII activity in the sample.

4 KIT COMPOSITION

Reagent 1 (2 vials) – REF 8010
Reagent 1 contains lyophilized bovine FX and a fibrin polymerization inhibitor.
Reagent 2 (2 vials) – REF 8020
Reagent 2 contains lyophilized human FIIa, human FIXa, calcium chloride and phospholipids.
FXa Substrate, 6 mL (2 vial) – REF 9080
Liquid solution of chromogenic FXa substrate (Z-D-Arg-Gly-Arg-pNA), 2.5 mmol/L, containing a thrombin inhibitor. Contains sodium azide ≤ 0.01% (≤0.1g/L)
Tris BSA Buffer, Stock Solution, 20 mL (1 vial) – REF 8050
Liquid stock solution of diluent buffer, containing 10% Bovine Serum Albumin (BSA) and a heparin antagonist. Stock solution contains sodium azide ≤ 0.01% (≤0.1g/L)

5 PRECAUTIONS AND WARNINGS

The reagents are matched – only use reagents from the same kit lot.

Human Factor IXa and thrombin were prepared from human plasma which was found to be negative when tested in accordance with current FDA required tests. Bovine serum albumin and bovine FX were prepared from bovine plasma from animals free from BSE.
However, no known test method can offer complete assurance that components derived from human or bovine blood will not transmit infectious agents, therefore, the handling and disposal of the reagents should be made with the required caution, as being potentially infectious[1].

Sodium azide may react with lead and copper plumbing to form explosive metal azide. Always flush with large volumes of water when discarding into a sink.

6 PREPARATION

Reagent 1
Reconstitute with **6.0 mL** water. Allow to stand for at least 30 min at 15-25°C with intermittent gentle mixing for complete reconstitution. Homogenize the content gently before use.
Reagent 2
Reconstitute with **6.0 mL** water. Allow to stand for at least 30 min at 15-25°C with intermittent gentle mixing for complete reconstitution. Homogenize the content gently before use.
FXa Substrate, 6 mL
Ready for use.
Tris BSA Buffer, Stock Solution, 20 mL
Dilute 1 + 9 with water to obtain a 0.05 mol/L Tris-HCl buffer working solution, pH 7.3 (at 20°C), with 1% bovine serum albumin and a heparin antagonist.
NB: The vial is slightly over dispensed. Always measure up the desired volume prior to 10-fold dilution with water.

Note: All reconstitutions and dilutions should be made with water of a quality of at least NCCLS Type II water or Ph. Eur. water for injection.

7 STORAGE AND STABILITY

The sealed reagents are stable at 2-8°C until the Expiry Date printed on the label. Opened vials must be handled with care to avoid contamination during use.

Homogenize the content gently before each use.

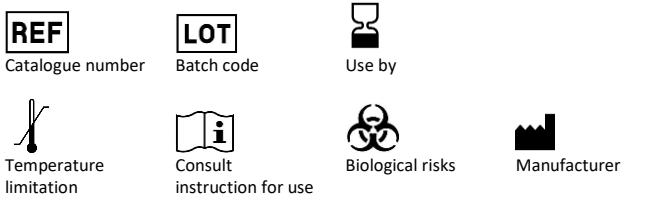
- **Reagent 1:** Stability after reconstitution is 72 hours (h) at 2-8°C, 24h at 15-25°C, 2h at 37°C and 12 months at ≤ -70°C.
- **Reagent 2:** Stability after reconstitution is 72 hours (h) at 2-8°C, 24h at 15-25°C, 2h at 37°C and 12 months at ≤ -70°C.
- **Chromogenic FXa substrate:**
  Opened vial is stable for 12 months at 2-8°C, 12 months at <-20°C or
  7 days at 18-25°C. If the substrate becomes yellow it indicates the presence of a contaminant and the vial must be rejected.
- **Tris BSA Diluent Buffer**
  Stock Solution: Opened vial is stable for 12 months at 2-8°C provided microbial contamination is avoided.
  Buffer working solution should be used the same day as prepared.

8 MATERIALS REQUIRED BUT NOT PROVIDED

- Deionized water, NCCLS Type II water or Ph. Eur. Water for injection or higher quality
- For calibration: Human plasma or FVIII concentrate, traceable to a WHO International Standard for FVIII activity
- Citric acid, 2% (for end-point method)
- Calibrated pipettes
- Photometer, 405 nm (and 490 nm for end-point method)
- Heat incubator 37°C
- Plastic test tubes
- Stop-watch
- Vortex mixer

For microplate assay, make sure to use low binding microplates.

9 SYMBOLS USED



10 SPECIMEN COLLECTION AND TREATMENT

Sample collection must be in conformity with the recommendations for haemostasis tests. Freshly drawn venous blood (9 volumes) is collected into 0.109 M trisodium citrate anticoagulant (1 volume). Use silicon glass or a plastic test tube. Centrifuge for 15 min at 2000-2500 g. Refer to CLSI guideline H21-A5 for further instruction on specimen collection, handling and storage.

11 QUALITY CONTROL

Quality control plasmas with assigned FVIII activity are commercially available and should be used for validating the calibration curve. Normal and abnormal controls are recommended for a complete quality control program. The controls should be processed as the samples. Each laboratory should determine its own quality control range, either by means of the target values and ranges provided by the manufacturer of the controls or by means of its own confidence level established in the laboratory.

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12 METHOD - PLASMA

A calibration curve should be included in each run. A normal human plasma traceable to an International Standard should be used as calibrator.

Prepare standard dilutions in Tris BSA buffer working solution to obtain standards in the range 0 - 200% where 200% FVIII activity corresponds to dilution 1:30 of a plasma calibrator containing 1 IU/ml FVIII and 100% corresponds to dilution 1:60.

Prepare all dilutions in plastic test tubes.

SPECIAL NOTE:
In order to get the full assay performance, the calibration curve and sample dilutions must be prepared just before running the assay to avoid FVIII degradation which could lead to erroneous results.

The calibration curve may be prepared as below. If only samples with low FVIII activities are analyzed, 200% and 150% can be replaced with 10% and 5% standards (total dilution 1:600 and 1:1200 respectively):

Preparation of FVIII Calibration curve, RANGE 0 - 200 %			
FVIII Standard %	**Total Dilution**	**Volume**	**Tris BSA Buffer, working solution**
Predilution	1:10	100 µL of plasma	900 µL
200%	1:30	100 µL of predilution	200 µL
150%	1:40	100 µL of predilution	300 µL
100%	1:60	100 µL of predilution	500 µL
50%	1:120	100 µL of predilution	1100 µL
20%	1:300	50 µL of predilution	1450 µL
0%		0	500 µL

NOTE: 100% activity is defined as a FVIII activity of 1 IU/mL in plasma. In case the FVIII activity of the plasma standard differs from this value, the appropriate correction factor should be used when calculating the sample result. It is recommended to express all sample results as IU/mL.

Sample dilution - Samples with expected activity 5 - 200%
Plasma samples with an estimated potency of **5 - 200 %** (0.05 - 2 IU/mL) should be analysed using sample dilution **1:60.** The FVIII activity of the tested sample is obtained directly from the calibration curve.
Sample dilution - Samples with expected activity 0 - 5%
Plasma samples with an estimated potency of **0 - 5%** (0 – 0.05 IU/mL) should be analysed using sample dilution **1:15. The FVIII activity of the tested sample is obtained by multiplying the results directly obtained from the calibration curve with the factor x0.25.**

Sample Blank (End-point method only)
Sample blanks are in general not necessary but should be included when analyzing hemolytic, icteric or lipemic plasma samples, using the end-point method. A sample blank is obtained by adding citric acid prior to the other reagents. Deduct the sample blank absorbance from the sample absorbance prior to calculating the result.

13 METHOD – CONCENTRATES

FVIII concentrates could be analyzed by first prediluting the sample to 0.2 - 2 IU/ml followed by a dilution of 1:60 in Tris BSA Buffer working solution. The European Pharmacopoeia recommends parallel line analysis for potency assignment of biological samples[2] and that therapeutic FVIII concentrates are prediluted in Haemophilia A plasma, or in an artificially prepared reagent that contains sufficient von Willebrand factor and that gives results that do not differ significantly from those obtained employing haemophilia plasma.[3]

14 ASSAY PROTOCOL– PLASMA AND CONCENTRATES

14.1 Manual method

Sample / Standard dilution	**50 µL**
Reagent 1 (Preincubated at 37°C)	**50 µL**
Reagent 2 (Preincubated at 37°C)	**50 µL**
Activation - Mix and incubate for 3 min at 37°C	
FXa Substrate (Preincubated at 37°C)	**50 µL**
Kinetic method: Read ΔA405/min at 37°C *End-point method: Hydrolysis at 37°C for 5 min*	
Citric acid, 2% (End-point method only)	**50 µL**

Kinetic reading:
Read the absorbance at 405 nm and record the change in absorbance.
End-point method:
Stop the reaction with 2% citric acid. Read the absorbance at 405 nm, using 490 nm as reference wavelength. Absorbance readings should be made within 2 hours after termination of the substrate hydrolysis.

14.2 Automated methods

Protocols for various automated coagulation instruments are available upon request. Contact Rossix at info@rossix.com for help with setting up the assay on automated instruments.
For application on an instrument, always adhere to the established assay concentrations of the reagents during activation and to the established sample dilutions.

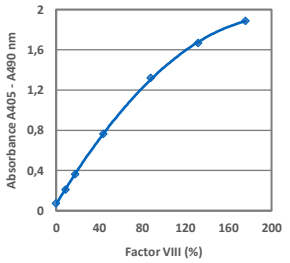
15 CALCULATION

Plasmas:

- Plot the maximal absorbance change/minute ($\Delta A405_{max}$/min) or absorbance (A405-490) vs. FVIII activity in a linear graph. Use a quadratic curve fit.
- The FVIII activity of the tested sample is obtained directly from the calibration curve (Samples diluted 1:60) or by multiplying with the factor 0.25 (Samples diluted 1:15). Correct the obtained value with the appropriate correction factor if the FVIII activity of the normal plasma standard differs from 1 IU/mL.
- Adjust for the dilution factor if several dilutions are used.
- Express the sample result as IU/mL or %.

16 EXAMPLE OF CALIBRATION CURVE

Example of calibration curve obtained using the manual end point method and 5 min hydrolysis time. This calibration curve is an example only and should not be used for calculating sample results. Always include a calibration curve in each assay series.



17 EXPECTED VALUES

The normal range for Factor VIII activity is 0.5 – 1.5 IU/mL [4]
FVIII Deficiency, also known as Haemophilia A, can be divided into three categories[5]: Mild (0.05 - 0.4 IU/mL), moderate (0.01 - 0.05 IU/mL) and severe (≤0.01 IU/mL). FVIII levels can also be decreased in patients with hepatic disease, cirrhosis and DIC.

18 PERFORMANCE CHARACTERISTICS

Results obtained using the manual microplate method
Detection limit: 0.3% (0.003 IU/mL), calculated according to CLSI EP17-A using sample dilution of 1:15.
Quantification limit: 0.7% (0.007 IU/mL), calculated according to CLSI EP17-A using sample dilution of 1:15.
Precision:
Repeatability (Intra assay CV): **≤4%**
Within Laboratory (Inter assay CV): **≤4%**
The precision was determined at 5%, 90% and 130% Factor VIII activity.
Linearity: 0.5 - 200% (0.005 – 2 IU/mL), calculated according to CLSI EP06-A.

19 REFERENCES

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