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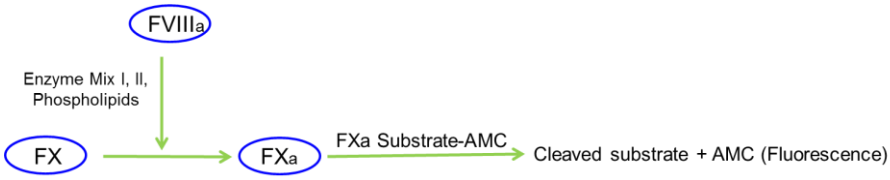
Factor VIIIa Activity Assay Kit (Fluorometric)

(Catalog # K358-100, 100 assays, Store kit at -20°C)

I. Introduction:

The coagulation Factor VIII, also known as anti-hemophilic factor (AHF) is a vital blood-clotting protein. Factor VIII circulates in the bloodstream as an inactive protein, bound to a large multimeric glycoprotein called von Willebrand factor (VWF). Upon an injury to the blood vessel, VWF dissociates from FVIII and releases the active form of FVIII (FVIIIa). In the presence of calcium ions and negatively charged membrane phospholipids, activated factor VIII (FVIIIa) then binds to the activated Factor IX (FIXa) and proteolytically activates factor X (FX) to factor Xa (FXa). BioVision's Factor VIIIa Activity Assay kit is based on the ability of FVIIIa to generate FXa. The generated FXa proteolytically cleaves a synthetic substrate and releases a fluorophore, AMC, which can be easily quantified by fluorescence microplate reader. The assay is simple, rapid and can detect activity as low as 1 ng of FVIIIa in a variety of samples.



II. Applications:

- Determine activity of pure FVIIIa
- Detect activity of FVIIIa in plasma, and serum

III. Sample Type:

- Purified enzyme
- Serum, plasma

IV. Kit Contents:

Components	K358-100	Cap Code	Part Number
FVIIIa Assay Buffer	15 ml	WM	K358-100-1
FXa Substrate-AMC	0.2 ml	Red	K358-100-2
Enzyme Mix I	1 vial	Blue	K358-100-3
Enzyme Mix II	1 vial	Purple	K358-100-4
Phospholipids	0.6 ml	Black	K358-100-5
FVIIIa Enzyme Standard (2.6 µg)	1 vial	Green	K358-100-6

V. User Supplied Reagents and Equipment:

- 96-well white microplate with flat bottom (cat. no. 2000)
- Multi-well spectrophotometer.

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge small vials at low speed prior to opening. Read entire protocol before performing the assay.

- **FVIIIa Assay Buffer:** Bring to room temperature before use.
- **Enzyme Mix I:** Reconstitute in 220 µl FVIIIa Assay Buffer. Mix well by pipetting up and down. Briefly centrifuge, aliquot and store at -20°C. Avoid repeated freeze/thaw.
- **Enzyme Mix II:** Reconstitute in 220 µl FVIIIa Assay Buffer. Mix well by pipetting up and down. Briefly centrifuge, aliquot & store at -80°C. Avoid repeated freeze/thaw.
- **Phospholipid:** Vortex for 10 sec. before each use. Phospholipids can be stored at 4°C for one month. For long term storage -20°C is recommended. Avoid repeated freeze/thaw.
- **FVIIIa Enzyme Standard:** Reconstitute in 52 µl FVIIIa Assay Buffer to prepare a stock solution of 50 ng/µl. Mix well by pipetting up and down. Aliquot and store at -80°C. Avoid repeated freeze/thaw.

VII. Factor VIIIa Activity Assay Protocol:

1. **Sample Preparation:** Dilute serum and plasma samples 10 times with FVIIIa Assay Buffer and add 2-5 µl/well into a 96-well plate in two wells (Sample Well [**S**] and Background Control Well [**Bck**]). For purified enzyme, add 2-5 µl (in the expected range of 0.2 to 2 ng) per well into desired well(s). Adjust the volume of background control and sample wells to 10µl/well with FVIIIa Assay Buffer.

   Notes:

   a. For unknown samples, we suggest doing pilot experiment and testing several amounts of FVIIIa to ensure the readings are within the Standard Curve range.

   b. Background control well is necessary to subtract basal Factor VIIIa activity that might be present in the sample.

2. **Standard Curve:** Prepare FVIIIa Enzyme Working Solution (0.5 ng/µl) just before use by adding 2 µl of FVIIIa Enzyme stock solution (50 ng/µl) to 198 µl of FVIIIa Assay Buffer. Add 0, 2, 4, 6, 8, and 10 µl of FVIIIa Enzyme working solution (0.5 ng/µl) into a series of

wells in a 96-well plate to prepare 0, 1, 2, 3, 4, and 5 ng/well of FVIIIa Enzyme Standard. Adjust the volume of all Standard wells to 10µl with FVIIIa Assay Buffer.

Note: Store FVIIIa Enzyme Working Solution at -80°C and use within a week.

3. **Reaction Mix**: Prepare a master mix of 10 µl/well by adding the components in the order shown:

Enzyme Mix I	2 µl
Phospholipids	6 µl
Enzyme Mix II	2 µl

Mix and add 10 µl of the master mix into each standard and sample well. Add 10 µl of FVIIIa Assay Buffer to background control well(s). Adjust the volume to 98 µl/well with FVIIIa Assay Buffer. Mix well by pipetting up and down. Incubate for 15 min. at 37°C. After incubation, add 2 µl of FXa substrate-AMC into Standard, background control and sample wells. Mix well.

4. **Measurement:** Measure fluorescence in kinetic mode for 30-60 min. at 37°C (Ex/Em = 360/450 nm). Choose two time points (T_1 and T_2) in the linear range of the plot and obtain the corresponding values for the fluorescence ($RFUs_1$ and $RFUs_2$ and RFU_{bck1} and $RFUbck_2$).

Note: Incubation time depends on the FVIIIa activity in the samples. We recommend measuring fluorescence in kinetic mode, and choosing two time points (T_1 and T_2) in the linear range to calculate the FVIIIa activity of the samples.

5. **Calculations**: Subtract 0 Standard reading from all readings. Plot the Factor VIIIa Standard Curve. Subtract the background signal from the sample signal. Apply sample's ΔRFU to Factor VIIIa Standard Curve to obtain corresponding Factor VIIIa (B, in ng) and calculate the activity of Factor VIIIa in the sample as:

$$\textbf{Sample Factor VIIIa Activity} = \frac{\boldsymbol{B}}{\mathbf{V}} \times \boldsymbol{Dilution\ Factor} = \frac{\boldsymbol{ng}}{\boldsymbol{ml}} = \frac{\mu\boldsymbol{g}}{\boldsymbol{L}}$$

Where, **B** is FVIIIa amount in the sample well from Standard Curve (ng)
V is sample volume added into the reaction well (ml)

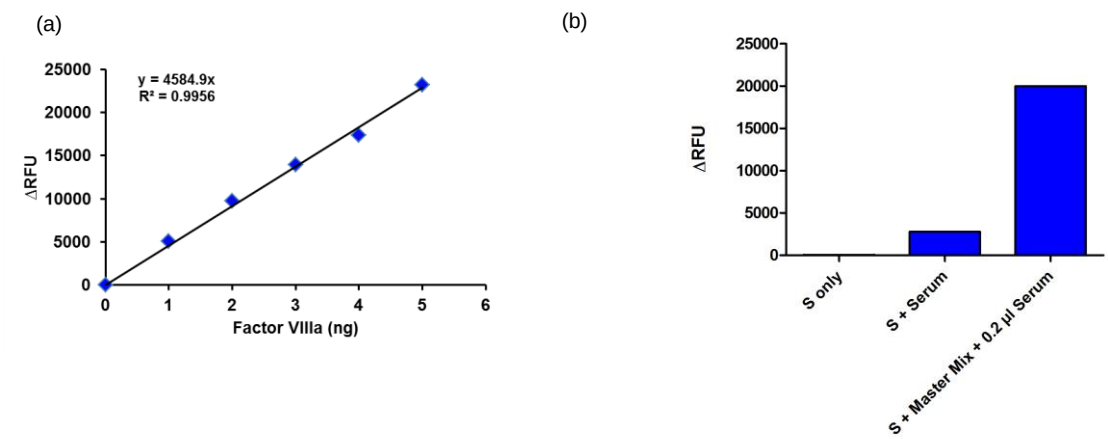


Figure: (a) Standard plot of Factor VIIIa activity. (b) Factor VIIIa activity was measured in serum samples in the presence and absence of the master mix. S: Substrate. Assays were performed following the kit protocol.

VIII. RELATED PRODUCTS:

Factor IXa Activity Fluorometric Assay Kit (K364)
Factor Xa Inhibitor Screening Kit (Fluorometric) (K362)
Thrombin Activity Fluorometric Assay Kit (K373)
Plasmin Activity Assay Kit (Fluorometric) (K381)
Plasmin Sepharose Beads (7926)
Urokinase, human recombinant (7696)
Factor Xa Activity Fluorometric Assay Kit (K361)
Factor Xa, Human Plasma (7689)
Thrombin Inhibitor Screening Kit (Fluorometric) (K374)
Plasmin Inhibitor Screening Kit (Fluorometric) (K382)
Urokinase Sepharose Beads (7927)
Urokinase Activity Fluorometric Assay Kit (K728)

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