ab204696

Factor VIIIa Activity Assay Kit (Fluorometric)

Instructions for Use

For rapid, sensitive and accurate detection of Factor VIII activity.

This product is for research use only and is not intended for diagnostic use.

PLEASE NOTE: With the acquisition of BioVision by Abcam, we have made some changes to component names and packaging to better align with our global standards as we work towards environmental-friendly and efficient growth. You are receiving the same high-quality products as always, with no changes to specifications or protocols.

Version 4 Last Updated 30 October 2023

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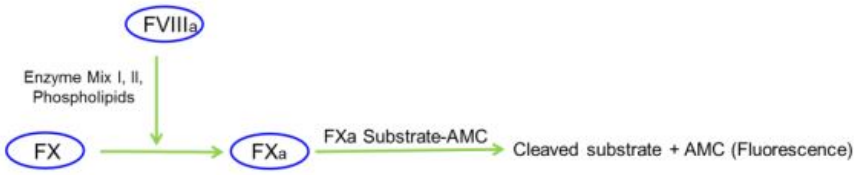
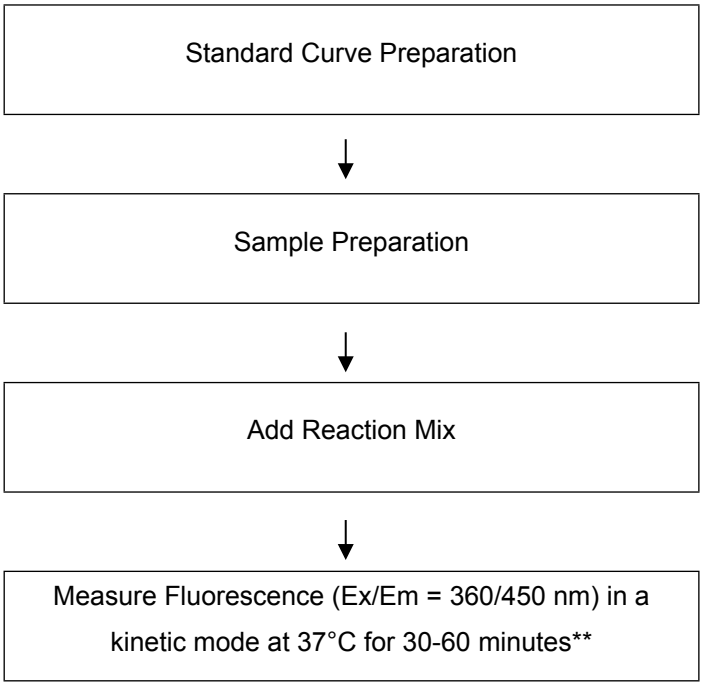
1. BACKGROUND

Factor VIIIa Activity Assay Kit (Fluorometric) (ab204696) 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.



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).

2. ASSAY SUMMARY



**For kinetic mode detection, incubation time given in this summary is for guidance only.*

3. PRECAUTIONS

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

4. STORAGE AND STABILITY

Store kit at -20ºC in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in Materials Supplied section.

Aliquot components in working volumes before storing at the recommended temperature. **Reconstituted components are stable for 2 months.**

5. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Assay Buffer XLIV/FVIIIa Assay Buffer	15 mL	-20°C	-20°C
FXa Substrate/FXa Substrate-AMC	200 µL	-20°C	-20°C
Enzyme Mix XXIII/Enzyme Mix I	1 Vial	-20°C	-20°C
Enzyme Mix XXII/Enzyme Mix II	1 Vial	-20°C	-80°C
Phospholipid Mixture/Phospholipids	600 µL	-20°C	-20°C
FVIIIa Enzyme Standard	2.6 µg	-20°C	-80°C

7. MATERIALS REQUIRED, NOT SUPPLIED

These materials are not included in the kit, but will be required to successfully perform this assay:

- Microcentrifuge
- Vortex
- Pipettes and pipette tips
- Fluorescent microplate reader – equipped with filter Ex/Em = 360/450 nm
- 96 well plate with clear flat bottom preferably white
- Heat block or water bath

8. TECHNICAL HINTS

- **This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.**
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Keep enzymes, heat labile components and samples on ice during the assay.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on.

9. REAGENT PREPARATION

- Briefly centrifuge small vials at low speed prior to opening.

9.1 **Assay Buffer XLIV/FVIIIa Assay Buffer:**

Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

9.2 **Enzyme Mix XXIII/Enzyme Mix I:**

Reconstitute in 220 µL Assay Buffer XLIV/FVIIIa Assay Buffer. Mix well by pipetting up and down, and briefly centrifuge the vial. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Store at -20°C. Avoid repeated freeze/thaw. Keep on ice while in use.

9.3 **Enzyme Mix XXII/Enzyme Mix II:**

Reconstitute in 220 µL Assay Buffer XLIV/FVIIIa Assay Buffer. Mix well by pipetting up and down, and briefly centrifuge the vial. Aliquot enzyme so that you have enough volume to perform the desired number of assays. Avoid repeated freeze/thaw. Store at -80°C. Keep on ice while in use.

9.4 **Phospholipid Mixture/Phospholipid Vesicles:**

Vortex for 10 seconds before each use. Phospholipid Mixture/Phospholipids can be stored at 4°C for one month. For long term storage -20°C is recommended. Avoid repeated freeze/thaw.

9.5 **FVIIIa Enzyme Standard (2.6 µg):**

Reconstitute in 52 µL Assay Buffer XLIV/FVIIIa Assay Buffer to prepare a stock solution of 50 ng/µL. Mix well by pipetting up and down. Aliquot reconstituted standard so that you have enough volume to perform the desired number of assays. Avoid repeated freeze/thaw. Store at -80°C.

9.6 **FXa Substrate/FXa Substrate-AMC:**

Ready to use as supplied. Aliquot FXa Substrate/substrate so that you have enough volume to perform the desired

number of assays. Avoid repeated freeze/thaw. Store at -20°C.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted FVIIIa Standard solution can be stored at -80°C for future use. Use within a week.

10.1 Prepare 200 µL of 0.5 ng/µL FVIIIa Enzyme Standard by diluting 2 µL of the provided FVIIIa Enzyme stock solution (50 ng/µL) with 198 µL of Assay Buffer XLIV/FVIIIa Assay Buffer.

10.2 Using 0.5 ng/µL FVIIIa Enzyme Standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes.

Standard #	Volume of Standard (µL)	Assay Buffer XLIV/Assay Buffer (µL)	Final volume standard in well (µL)	End Conc FVIIIa in well (ng/well)
1	0	30	10	0
2	6	24	10	1
3	12	18	10	2
4	18	12	10	3
5	24	6	10	4
6	30	0	10	5

Each dilution has enough amount of standard to set up duplicate readings (2 x 10 µL).

11. SAMPLE PREPARATION

General Sample information:

- We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.
- We recommend that you use fresh samples. Alternatively, if that is not possible, we suggest that you snap your samples in liquid nitrogen upon extraction and store the samples immediately at -80°C. When you are ready to test your samples, thaw them on ice. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

11.1 Plasma and Serum:

Dilute plasma and serum samples 10X with Assay Buffer XLIV/FVIIIa Assay Buffer before adding sample to microplate wells.

However, to find the optimal values and ensure your readings will fall within the standard values, we recommend performing several dilutions of the sample.

11.2 Purified enzyme:

Dilute purified enzyme in Assay Buffer XLIV/FVIIIa Assay Buffer to a final range of 0.1 – 0.4 ng/µL.

However, to find the optimal values and ensure your readings will fall within the standard values, we recommend performing several dilutions of the sample.

NOTE: *We suggest using different dilutions of sample to ensure readings are within the Standard Curve range.*

12. ASSAY PROCEDURE and DETECTION

- **Equilibrate all materials and prepared reagents to room temperature prior to use.**
- **It is recommended to assay all standards, controls and samples in duplicate.**
- **Prepare all reagents, working standards, and samples as directed in the previous sections.**

12.1 **Set up Reaction wells:**

- Standard wells = 10 µL standard dilutions.
- Sample wells = 2 – 5 µL samples (adjust volume to 10 µL/well with Assay Buffer XLIV/FVIIIa Assay Buffer).
- Background control sample wells= 2 - 5 µL samples (adjust volume to 10 µL/well Assay Buffer XLIV/FVIIIa Assay Buffer).

12.2 **Reaction Mix:**

Prepare 10 µL of Reaction Mix for each reaction:

Component	Reaction Mix (µL)
Enzyme Mix XXIII/Enzyme Mix I	**2**
Phospholipid Mixture/Phospholipids	**6**
Enzyme Mix XXII/Enzyme Mix II	**2**

Mix enough reagents for the number of assays (samples and controls) to be performed. Prepare a master mix of the Reaction Mix to ensure consistency. We recommend the following calculation: X µL component x (Number reactions +1)

12.3 Add 10 µL of Reaction Mix into each standard and sample well. Mix well.

12.4 Add 10 µL of Assay Buffer XLIV/FVIIIa Assay Buffer to all background control well(s). Mix well.

12.5 Adjust the volume to 98 µL/well with Assay Buffer XLIV/FVIIIa Assay Buffer. Mix well.

12.6 Incubate for 15 minutes at 37°C.

12.7 Add 2 µL of FXa Substrate/FXa substrate-AMC into all wells (standard, background control and sample wells). Mix well.

12.8 Measure output on a fluorescent microplate reader at Ex/Em = 360/450 nm in a kinetic mode, every 2 – 3 minutes, for 30 – 60 minutes at 37°C protected from light.

NOTE: *Sample incubation time can vary depending on FVIIIa activity in the samples. We recommend measuring fluorescence in kinetic mode and then choosing two time points (T_1 and T_2) during the linear range.*

RFU value at T_2 should not exceed the highest RFU in the standard curve. For standard curve, do not subtract RFU_1 from RFU_2 reading.

13. CALCULATIONS

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer and reanalyzed, then multiplying the concentration found by the appropriate dilution factor.
- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

13.1 Average the duplicate reading for each standard and sample.

13.2 If the sample background control is significant, then subtract the sample background control from sample reading.

13.3 Subtract the mean fluorescence value of the blank (Standard #1) from all standard and sample readings. This is the corrected fluorescence.

13.4 Plot the corrected fluorescence values for each standard as a function of the final concentration of FVIIIa Enzyme.

13.5 Draw the best smooth curve through these points to construct the standard curve. Calculate the trend line equation based on your standard curve data (use the equation that provides the most accurate fit).

13.6 Activity of FVIIIa is calculated as:

$$\mathbf{\Delta RFU_{360/450nm} = (RFU_2 - RFU_{2BG}) - (RFU_1 - RFU_{1BG})}$$

Where:

RFU_1 is the sample reading at time T_1.

RFU_{1BG} is the background control sample at time T_1.

RFU_2 is the sample reading at time T_2.

RFU_{2BG} is the background control sample at time T_2.

13.7 Use the $\Delta RFU_{360/450nm}$ to obtain B (in ng) of Factor VIIIa.

13.8 Activity of Factor VIIIa in the test is calculated as:

$$FVIIIa\ Activity = \left(\frac{B}{V}\right) * Dilution\ Factor = ng/mL = \ \mu g/L$$

Where:

B = Amount of FVIIIa from Standard Curve (ng).

V = Original sample volume added into the reaction well (in mL).

14. TYPICAL DATA

TYPICAL STANDARD CURVE – Data provided for **demonstration purposes only**. A new standard curve must be generated for each assay performed.

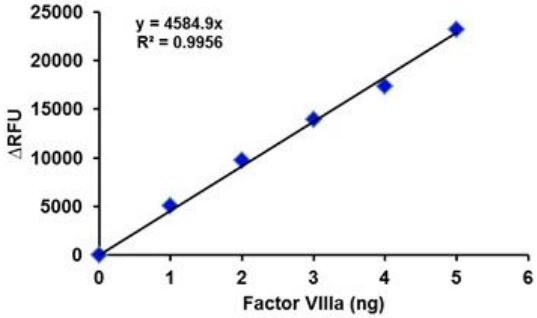


Figure 1 Typical FVIIIa Standard calibration curve using fluorometric reading.

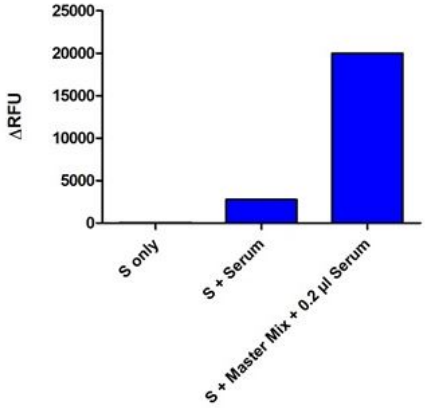


Figure 2. Factor VIIIa activity was measured in serum samples in the presence and absence of the master mix. S: Substrate.

15. QUICK ASSAY PROCEDURE

***NOTE**: This procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.*

- Prepare standard and prepare enzyme mix; get equipment ready.
- Prepare appropriate standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for standard (10 µL), samples (10 µL) and background wells (10 µL).
- Prepare Factor VIII Reaction Mix (Number reactions + 1).

Component	Reaction Mix (µL)
Enzyme Mix XXIII/Enzyme Mix I	**2**
Phospholipid Mixture/Phospholipids	**6**
Enzyme Mix XXII/Enzyme Mix II	**2**

- Add 10 µL of Factor VIII Reaction Mix to the standard, sample wells.
- Add 10 µL of Assay Buffer XLIV/FVIIIa Assay Buffer to background control wells.
- Adjust the volume to 98 µL/well with Assay Buffer XLIV/FVIIIa Assay Buffer. Mix well.
- Incubate for 15 minutes at 37°C.
- Add 2 µL of FXa Substrate/FXa substrate-AMC into Standard, background control and sample wells. Mix well.
- Incubate plate at 37°C during 30-60 minutes and read fluorescence at Ex/Em= 360/450 nm in a kinetic mode.

16. TROUBLESHOOTING

Problem	Cause	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at room temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different 96-well plate	Colorimetric: Clear plates Fluorometric: black wells/clear bottom plate
Sample with erratic readings	Samples not deproteinized (if indicated on protocol)	Use PCA precipitation protocol for deproteinization
	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at -80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/ Higher readings in samples and Standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol

Problem	Cause	Solution
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions on protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

17. INTERFERENCES

These chemicals or biological materials will cause interferences in this assay causing compromised results or complete failure.

- RIPA buffer – it contains SDS which can destroy/decrease the activity of the enzyme.

18. **FAQ**

19. **NOTES**

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)