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ab204727
Factor IXa Activity Assay Kit (Fluorometric)

Instructions for Use

For rapid, sensitive and accurate detection of Factor IXa 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 5 Last Updated 30 October 2023

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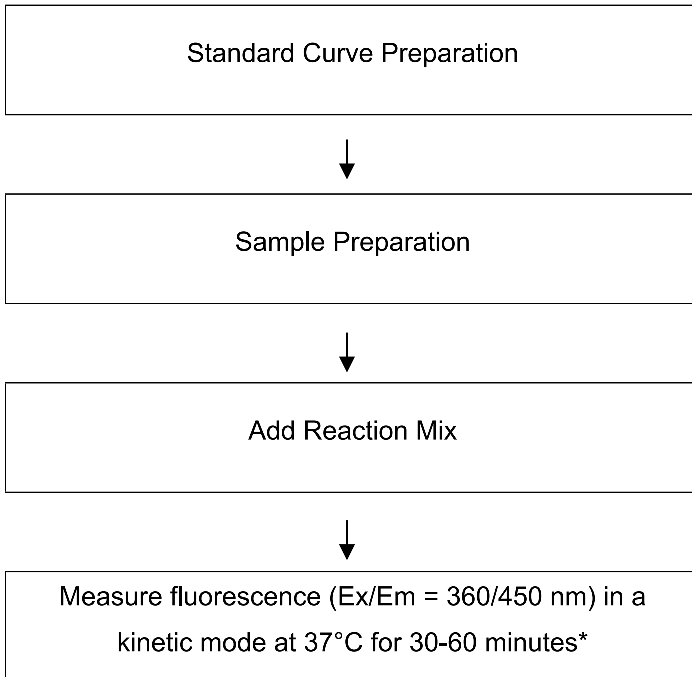
1. BACKGROUND

Factor IXa Activity Assay Kit (Fluorometric) (ab204727) is based on the ability of FIXa 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 10 pg of FIXa in a variety of samples.

The coagulation Factor IX (or Christmas factor, EC 3.4.21.22) is a vitamin K-dependent serine protease. Factor IX is produced as an inactive precursor and is activated via cleavage by either factor XIa (contact pathway) or factor VIIa (tissue factor pathway). In the presence of calcium ions and negatively charged membrane phospholipids, activated factor IX (FIXa) then binds to the activated Factor VIII (FVIIIa) 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/FIXa Assay Buffer	15 mL	-20°C	-20°C
FXa Substrate/FXa Substrate-AMC	200 µL	-20°C	-20°C
Active Factor VIIIa/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
FIXa Enzyme Standard	10 ng	-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
- Multi-well spectrophotometer
- 96 well Black microplate with flat bottom
- 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/FIXa Assay Buffer:**

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

9.2 **Active Factor VIIIa/Enzyme Mix I:**

Reconstitute Active Factor VIIIa/Enzyme Mix I in 220 µL Assay Buffer XLIV/FIXa Assay Buffer. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at -20°C. Keep on ice while in use.

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

Reconstitute Enzyme Mix XXII/Enzyme Mix II in 220 µL Assay Buffer XLIV/FIXa Assay Buffer. Aliquot standard so that you have enough volume to perform the desired number of assays. 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 **FIXa Enzyme Standard (10ng):**

Reconstitute in 20 µL Assay Buffer XLIV/FIXa Assay Buffer to prepare a 0.5 ng/µL standard stock solution. Aliquot standard so that you have enough volume to perform the desired number of assays. Store at -80°C. Keep on ice while in use.

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. Store at -20°C. Keep on ice while in use.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted standard solution is unstable and cannot be stored for future use.

10.1 Prepare 200 µL of 5 pg/µL FIXa Enzyme Standard by diluting 2 µL of the provided FIXa Enzyme stock solution (0.5 ng/µL) with 198 µL of Assay Buffer XLIV/FIXa Assay Buffer.

10.1.1 Using 5 pg/µL FIXa 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 FIXa Enzyme in well (pg/well)
1	0	10	10	0
2	2	8	10	10
3	4	6	10	20
4	6	4	10	30
5	8	2	10	40
6	10	0	10	50

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. If you cannot perform the assay at the same time, we suggest that you complete the Sample Preparation step before storing the samples. Alternatively, if that is not possible, we suggest that you snap freeze 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/FIXa 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/FIXa Assay Buffer to a final range of 5 – 10 pg/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 volumes 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 – 10 µL samples (adjust volume to 10 µL/well with Assay Buffer XLIV/FIXa Assay Buffer).
- Background control sample wells= 2 - 10 µL samples (adjust volume to 10 µL/well Assay Buffer XLIV/FIXa Assay Buffer).

12.2 **Reaction Mix:**

Prepare 10 µL of Reaction Mix for each reaction:

Component	Reaction Mix (µL)
Active Factor VIIIa/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 Adjust the volume to 98 µL/well with Assay Buffer XLIV/FIXa Assay Buffer. Mix well.

12.5 Incubate for 15 minutes at 37°C.

12.6 Add 2 µL of FXa Substrate/FXa substrate-AMC into Standard, and sample wells. Mix well.

12.7 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 FIXa 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.

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 value of the blank (Standard #1) from all standard and sample readings. This is the corrected signal.

13.4 Plot the corrected values for each standard as a function of the final concentration of FIXa 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 FIXa is calculated as:

$\Delta RFU_{360/450nm} = (RFU_2 – RFU_1)/(T_2 – T_1)_{unknown}$ / Slope of the standard curve = amount of enzyme in well.

Where:

RFU_1 is the sample reading at time T_1.

RFU_2 is the sample reading at time T_2.

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

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

$$FIXa\ Activity = \left(\frac{B}{V}\right) * D = pg/mL = \ ng/L$$

Where:

B = Amount of FIXa from Standard Curve (pg).

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

D = Sample dilution factor

14. TYPICAL DATA

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

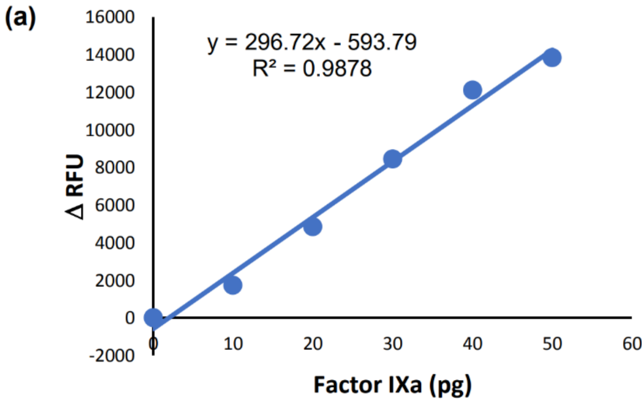


Figure 1 Typical FIXa Standard calibration curve.

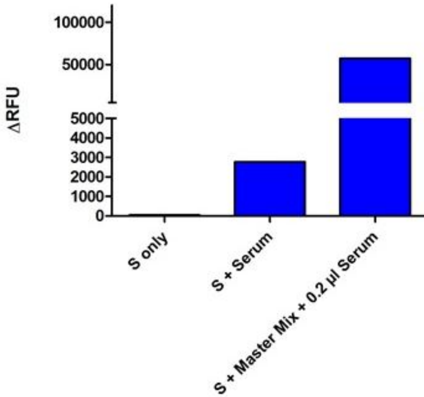


Figure 2. Factor IXa activity was measured in serum samples in the presence and absence of the master mix. S: Substrate.

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 IXa Reaction Mix (Number reactions + 1).

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

- Add 10 µL of Factor IX Reaction Mix to the standard, sample wells.
- Add 10 µL of Assay Buffer XLIV/FIXa Assay Buffer to background control well(s).
- Adjust the volume to 98 µL/well with Assay Buffer XLIV/FIXa Assay Buffer.
- Incubate for 15 minutes at 37°C.
- Add 2 µL of FXa Substrate/FIXa 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	Clear plates
Sample with erratic readings	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
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

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Technical Support

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