

ab204712

Factor Xa Inhibitor Screening Assay Kit (Fluorometric)

Instructions for Use

For rapid, sensitive and accurate screening of potential Factor Xa inhibitors.

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

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DATA ANALYSIS

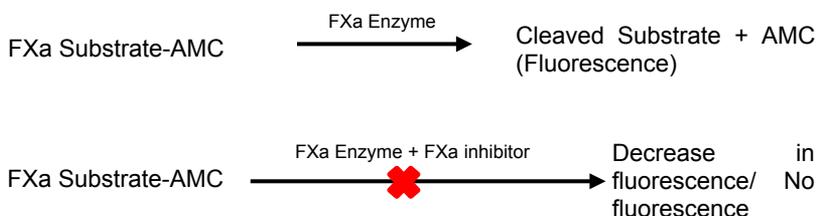
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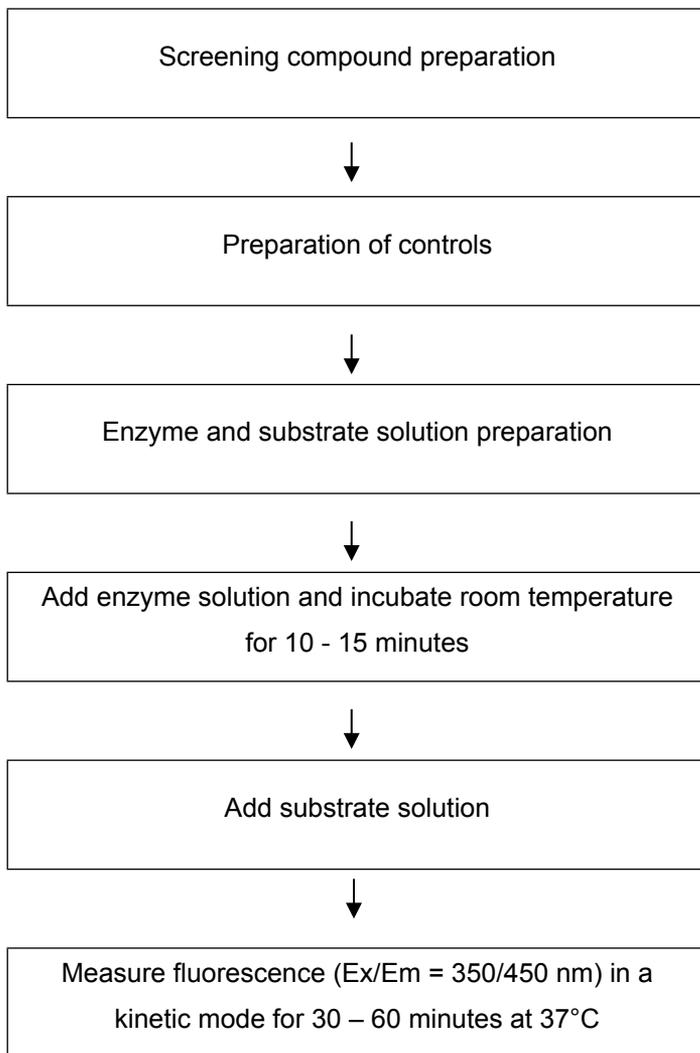
1. BACKGROUND

Factor Xa Inhibitor Screening Assay Kit (Fluorometric) (ab204712) is based on the ability of Factor Xa to cleave a synthetic substrate thereby releasing a fluorophore, AMC, which can be quantified by a fluorescence reader at Ex/Em = 350/450 nm. In the presence of an inhibitor, the extent of the cleavage reaction is reduced or completely abolished. The loss in the fluorescence intensity can be correlated to the amount of inhibitor present in the assay solution. The Factor Xa Inhibitor Screening Kit is a simple, straightforward, high-throughput assay to screen Factor Xa inhibitors.



Factor Xa (FXa) is the activated form of the coagulation factor X (Stuart-Power factor, thrombokinase, prothrombinase, thromboplastin, E.C.3.4.21.6). Factor X, a serine endopeptidase, plays an important role at several stages of the coagulation pathway. It acts by converting prothrombin into active thrombin by complexing with activated co-factor V in the prothrombinase complex. Unfractionated heparin and various low molecular weight heparins bind to plasma cofactor Antithrombin to inactivate several coagulation factors including factor Xa.

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 -20°C 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.

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)
FXa Dilution Buffer	1 mL	-20°C	-80°C
FXa Assay Buffer	15 mL	-20°C	-20°C
FXa Enzyme	5 µL	-20°C	-80°C
FXa Substrate	0.2 mL	-20°C	-20°C
FXa Inhibitor	10 µL	-20°C	-20°C

7. MATERIALS REQUIRED, NOT SUPPLIED

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

- Pipettes and pipette tips
- Microcentrifuge
- Fluorescent microplate reader – equipped with filter for Ex/Em = 350/450 nm
- 96 well plate: black plate 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 regulations.
- Keep enzymes and heat labile components and samples on ice during the assay.
- Make sure all buffers and developing solutions are at room temperature before starting the experiment.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the appropriate type of plate for the detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.

9. REAGENT PREPARATION

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

9.1 **FXa Assay Buffer:**

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

9.2 **FXa Dilution Buffer:**

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

9.3 **FXa Enzyme:**

Add 105 μ L of FXa Dilution Buffer to vial to prepare enzyme stock solution and mix well. Aliquot enzyme stock solution so that you have enough volume to perform the desired number of assays. Store at -80°C. Avoid repeated freeze/thaw.

9.4 **FXa Inhibitor:**

Ready to use as supplied. Equilibrate to room temperature before use. Aliquot inhibitor so that you have enough volume to perform the desired number of assays. Store at -20°C.

9.5 **FXa Substrate:**

Ready to use as supplied. Equilibrate to room temperature before use. Aliquot substrate so that you have enough volume to perform the desired number of assays. Store at -20°C.

10. SAMPLE PREPARATION

- Always prepare a fresh set of samples and controls for every use.

10.1 Screening Compounds:

10.1.1 Dissolve test compounds into appropriate solvent.

10.1.2 Dilute to 10X the desired test concentration with FXa Assay Buffer before use.

NOTE: *We suggest using different volumes of testing compounds if effective concentration is unknown.*

11. ASSAY PROCEDURE and DETECTION

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- It is recommended to assay all controls and samples in duplicate.

11.1 Set up reaction wells:

- Sample wells (S) = 10 μ L test inhibitors.
- Inhibitor Control wells (IC) = 1 μ L FXa Inhibitor + 9 μ L FXa Assay Buffer.
- Enzyme Control wells (EC) = 10 μ L FXa Assay Buffer.
- OPTIONAL: Solvent control (SC) = 10 μ L solvent. **NOTE:** solvents used to solubilize the inhibitors might affect the enzymatic activity therefore we recommend performing a solvent control well.

11.2 Prepare FXa Enzyme Solution:

Prepare 50 μ L of FXa Enzyme Solution for each well:

Component	Enzyme Solution (μ L)
FXa Assay Buffer	49
FXa Enzyme Stock Solution	1

Mix sufficient reagents for the number of assays to be performed. Prepare a master mix of the Enzyme Mix to ensure consistency. We recommend the following calculation: X μ L component x (Number reactions + 1).

- 11.3 Add 50 μ L of FXa Enzyme Solution to each well.
- 11.4 Incubate at room temperature for 10-15 minutes.

11.5 FXa Substrate Solution:

Prepare 40 μ L of FXa Substrate Solution for each well:

Component	Substrate Solution (μ L)
FXa Assay Buffer	38
FXa Substrate	2

ASSAY PROCEDURE and DETECTION

- 11.6 Add 40 μL of FXa Substrate Solution to each of S, EC, IC and SC wells.

The table below shows the set up reaction wells:

Component	Sample Well (S) (μL)	Inhibitor Control (IC) (μL)	Enzyme control (EC) (μL)	Solvent Control (SC) (μL)
Test Inhibitor compound	10	0	0	0
FXa Assay Buffer	0	9	10	0
FXa Inhibitor Control	0	1	0	0
Solvent test compound	0	0	0	10
FXa Enzyme solution	50	50	50	50
Fxa Substrate Solution	40	40	40	40

- 11.7 Measure fluorescence on a microplate reader at Ex/Em = 360/450 nm in a kinetic mode, every 2 – 3 minutes, for at least 30 – 60 minutes at 37°C.

NOTE: Incubation time depends on the FXa activity in samples. Longer incubation times may be required if FXa activity is low.

We recommend measuring the fluorescence in kinetic mode, and choosing two time points (T_1 & T_2) in the linear range to obtain the corresponding values for the fluorescence (RFU_1 and RFU_2) to calculate the FXa activity of the samples.

12. CALCULATIONS

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).
 - Average the duplicate reading for each test sample compound, Inhibitor Control and Enzyme control.
 - Choose two time points (T_1 and T_2) in the linear range of the plot and obtain the corresponding values for the fluorescence (RFU_1 and RFU_2).
 - Calculate the slope for all samples (S), Inhibition Control (IC) and Enzyme Control (EC) by dividing the net ΔRFU ($RFU_2 - RFU_1$) values with the time ΔT ($T_2 - T_1$).
 - Calculate the % Relative inhibitions as follows:

$$\% \text{ Relative Inhibition} = \frac{\text{Slope of EC} - \text{Slope of S}}{\text{Slope of EC}} \times 100$$

NOTE:

If RFU of SC < RFU of EC = make a higher stock of test inhibitor, or dissolve the inhibitor in lower concentration of the solvent; or use a different solvent.

If RFU of S < RFU of EC = treat as 100% inhibition and further dilute the test inhibitor and repeat the assay.

13. TYPICAL DATA

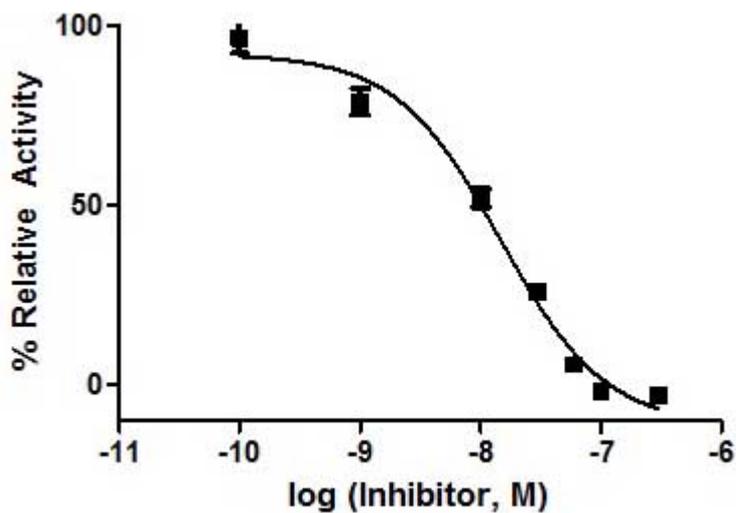


Figure 1. Inhibition of FXa Activity by the FXa Inhibitor provided in the kit.

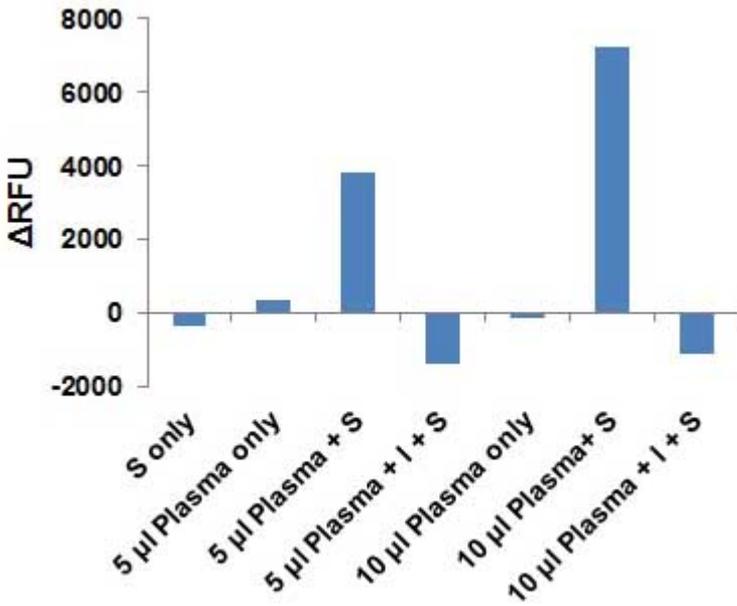


Figure 2. FXa Activity was measured in plasma samples in the presence or absence of FXa Inhibitor. S = Substrate, I = Inhibitor. Assay was performed following kit protocol.

14. 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 enzyme mix, substrate mix and get equipment ready.
- Prepare samples and dissolve test inhibitors in suitable solvent.
- Prepare Factor Xa Enzyme solution for all wells to be set up (50 μL /well)

Component	Enzyme Solution (μL)
Factor Xa Buffer	49
Factor Xa Enzyme Stock Solution	1

- Set up plate as follows:

Component	Sample Well (S) (μL)	Solvent control (SC) (μL)	Enzyme Control (EC) (μL)	Inhibitor Control (IC) (μL)
Enzyme Mix	50	50	50	50
Solvent test compound	0	10	0	0
Test Inhibitor Compound	10	0	0	0
Assay Buffer	0	0	10	9
Inhibitor control	0	0	0	1

- Incubate RT 10 – 15 min.
- Prepare 40 μL of Factor Xa Substrate Mix for each well

Component	Substrate Solution (μL)
Factor Xa Assay Buffer	38
Factor Xa Substrate	2

- Add 40 μL of Factor Xa Substrate Solution to each of S, EC, IC and SC wells.
- Measure plate in a kinetic mode at Ex/Em = 360/450 nm for 30-60 minutes at 37°C.

15. 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
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 μ L) 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

16. FAQ

17. NOTES

RESOURCES

Technical Support

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