ab197007
Thrombin Inhibitor Screening Assay Kit (Fluorometric)

Instructions for Use

For the rapid, sensitive and accurate screening of potential inhibitors of Thrombin.

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

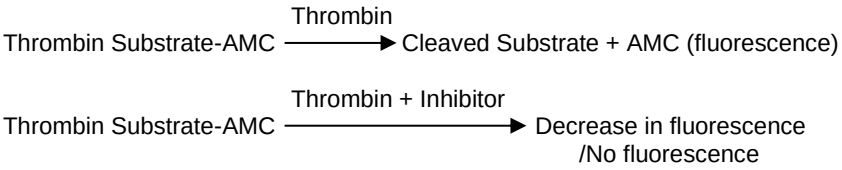
Version 3a, Last Updated 5 June 2025

Table of Contents

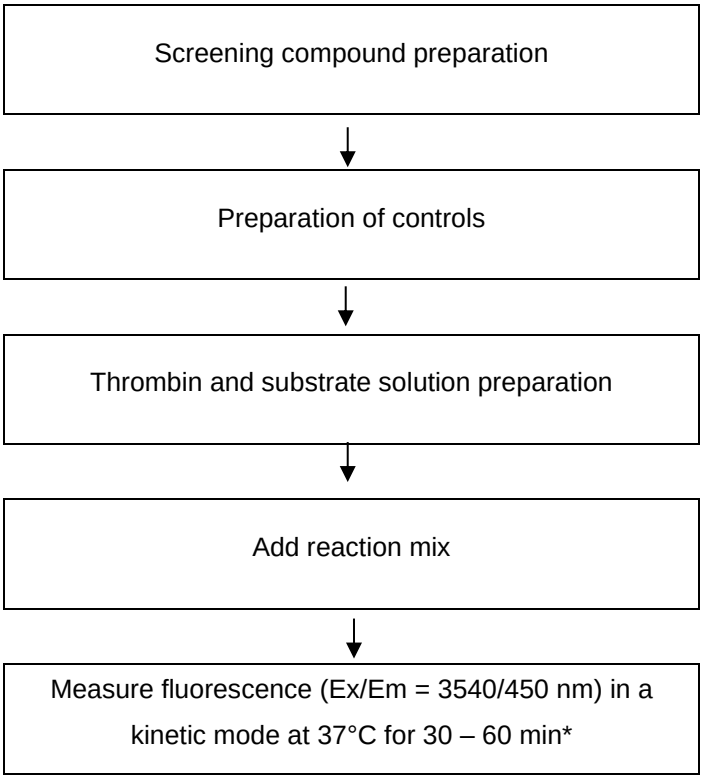
1. BACKGROUND

Thrombin Inhibitor Screening Assay Kit (fluorometric) (ab197007) utilizes the ability of thrombin to cleave a synthetic AMC-based peptide substrate to release AMC, which can be detected by measuring its fluorescence at Ex/Em = 350/450 nm. In the presence of thrombin specific inhibitors, the extent of 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 kit provides a simple and rapid method to screen potential inhibitors of thrombin.

Thrombin enzyme (Factor IIa) is an important clotting factor that controls the transformation of soluble fibrinogen to insoluble active fibrin strands. Thrombin is a serine protease that catalyzes many coagulation-related reactions. Thrombin inhibitors are used as anticoagulants to prevent arterial and venous thrombosis. Some of these inhibitors are currently in clinical use while others are in clinical development.



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 section 5.

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

5. MATERIALS SUPPLIED

Item	Amount	Storage Condition (Before Preparation)	Storage Condition (After Preparation)
Thrombin Dilution buffer	1 mL	-20°C	-20°C
Thrombin Assay Buffer	15 mL	-20°C	-20°C
Thrombin Standard	5 µL	-20°C	-20°C
Thrombin Substrate	0.5 mL	-20°C	-20°C
Thrombin Inhibitor	11 µL	-20°C	-20°C

6. MATERIALS REQUIRED, NOT SUPPLIED

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

- Microcentrifuge
- Pipettes and pipette tips
- Fluorescent microplate reader – equipped with filter for Ex/Em = 350/450 nm
- 96 well plate: black plates (clear flat bottoms) for fluorometric assay
- Heat block or water bath

7. LIMITATIONS

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not use kit or components if it has exceeded the expiration date on the kit labels.
- 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.

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.**
- 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.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Ensure plates are properly sealed or covered during incubation steps.
- Ensure complete removal of all solutions and buffers from tubes or plates during wash 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 **Thrombin Dilution Buffer:**

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

9.2 **Thrombin Assay Buffer:**

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

9.3 **Thrombin Standard:**

Dilute provided stock with 215 µL Thrombin Dilution Buffer. Mix well by pipetting up and down. Aliquot diluted enzyme so that you have enough volume to perform the desired number of tests. Avoid repeated freeze/thaw. Store at -80°C. Keep on ice while in use.

9.4 **Thrombin Substrate:**

Ready to use as supplied. Aliquot substrate so that you have enough volume to perform the desired number of tests. Store at -20°C. Keep on ice while in use.

9.5 **Thrombin Inhibitor**

9.6 **:**

Ready to use as supplied. Aliquot inhibitor so that you have enough volume to perform the desired number of tests. Store at -20°C. Keep on ice while in use.

10. SAMPLE PREPARATION

General Sample information:

- 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 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.

10.1 Plasma samples:

Plasma samples can be tested directly by adding sample to the 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.

10.2 Screening compounds:

Dissolve candidate inhibitors into proper solvent. Dilute to 10X the desired test concentration with Thrombin Assay Buffer.

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 **Prepare Enzyme Solution:**

Mix sufficient reagents for the number of assays to be performed. Prepare a master mix of the Enzyme Mix to ensure consistency.

For each well, prepare 50 µL of Thrombin Enzyme Solution.

Component	Enzyme Mix (µL)
Thrombin Assay Buffer	**48**
Diluted Thrombin Standard	**2**

11.2 **Set up Reaction wells:**

Set up Sample wells (S), Enzyme Control (EC) and Inhibitor Control (IC) wells as follows:

Component	Sample Well (S) (µL)	Solvent control (BC) (µL)	Enzyme control (EC) (µL)	Inhibitor Control (IC) (µL)
Test inhibitor compound	**10**		**0**	**0**
Solvent test compound		**10**		
Thrombin Assay Buffer	**0**		**10**	**9**
Thrombin Inhibitor	**0**		**0**	**1**

Incubate at room temperature for 10 – 15 minutes.

11.3 Add 50 µL of Thrombin Standard (Section 11.1) into each sample and control well.

11.4 Incubate at room temperature for 10 – 15 minutes.

11.5 **Substrate Mix:**

Prepare 40 µL of Substrate Mix for each reaction:

Component	Substrate Mix (µL)
Thrombin Assay Buffer	**35**
Thrombin Substrate	**5**

Mix enough reagents for the number of assays (samples and controls) to be performed. Prepare a master mix of the Substrate Mix to ensure consistency. We recommend the following calculation:

X µL component x (Number samples + controls +1).

11.6 Add 40 µL of Substrate Mix into each sample and control well.

11.7 Measure fluorescence on a microplate reader at Ex/Em = 350/450 nm in a kinetic mode, every 2 – 3 minutrs, for at least 30 – 60 minutes at 37°C protected from light.

NOTE: *Sample incubation time can vary depending on the Thrombin activity in samples. Longer incubation times may be required if Thrombin activity is low.*

We recommend measuring the RFU in kinetic mode, and choosing two time points (T_1 and T_2) in the linear portion of the time course to calculate the Thrombin activity.

Irreversible inhibitors that inhibit the Thrombin activity completely at the tested concentration will have ΔRFU = 0 and will show 100% relative inhibition.

12. CALCULATIONS

- For statistical reasons, we recommend each sample should be assayed with a minimum of two replicates (duplicates).

12.1 Average the duplicate reading for each sample, Inhibitor Control and Enzyme control.

12.2 Subtract the mean absorbance value of the Solvent Control (BC) if necessary from all controls and sample readings. This is the corrected absorbance.

12.3 Plot the corrected absorbance values for each sample and control as a function of the final concentration of compound.

12.4 Calculate the slope for all samples (S), Inhibition Control and Enzyme Control (EC):

$$Slope = \left(\frac{RFU2 - RFU1}{T2 - T1}\right) * D$$

Where:

RFU1 = Fluorescence value at Ex/Em = 350/450 nm at Time T_1.

RFU2 = Fluorescence value at Ex/Em = 350/450 nm at Time T_2.

T1 = Time of the first reading (A_1) in minutes.

T2 = Time of the second reading (A_2) in minutes.

D = sample dilution factor.

12.5 Calculate the % Relative inhibitions as follows

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

13. TYPICAL DATA

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

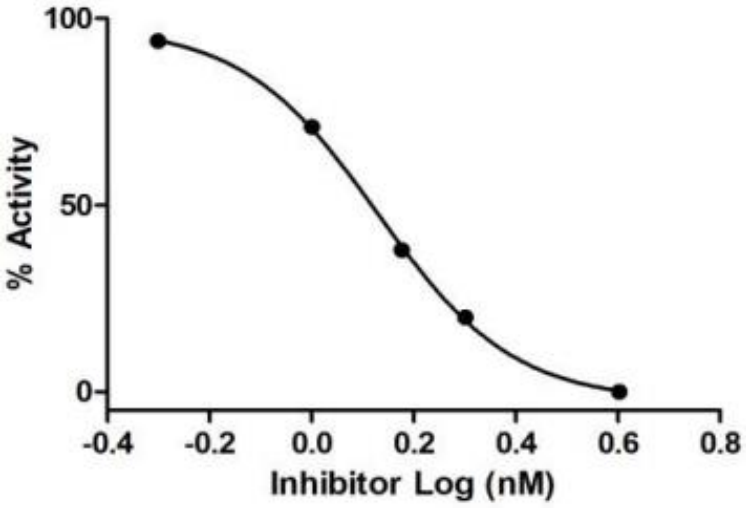


Figure 1: Inhibition of thrombin activity by a Thrombin Inhibitor.

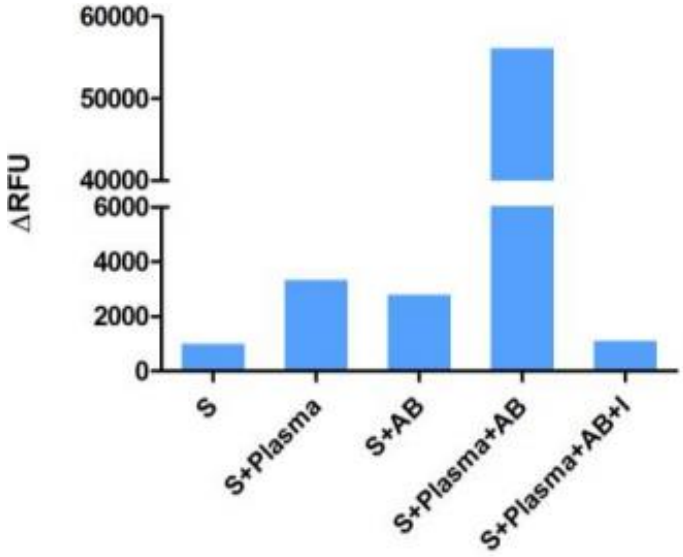


Figure 2: Thrombin activity was measured in plasma samples in the presence and absence of Thrombin Inhibitor. S = Substrate, I = Inhibitor, AB = Activation Buffer containing Factor Xa.

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, substrate, inhibitor, dilution buffer and assay buffer (aliquot if necessary); get equipment ready.
- Prepare samples and dissolve test inhibitors in suitable solvent.
- Prepare Enzyme Mix for all wells to be set up (50 µL/well).

Component	Enzyme Mix (µL)
Thrombin Assay Buffer	**48**
Diluted Thrombin Standard	**2**

- Set up plate as follows:

Component	Sample Well (S) (µL)	Enzyme Control (EC) (µL)	Inhibitor Control (IC) (µL)
Enzyme Mix	**50**	**50**	**50**
Test Inhibitor Compound	**10**	**0**	**0**
Thrombin Assay Buffer	**0**	**10**	**9**
Thrombin Inhibitor	**0**	**0**	**1**

- Incubate RT 10-15 min.
- Prepare 40 µL Thrombin Substrate Mix for each well (Number samples + controls + 1).

Component	Substrate Mix (µL)
Thrombin Assay Buffer	**35**
Thrombin Substrate	**5**

- Add 40 µL of Thrombin Substrate Mix to all wells.
- Measure plate on a microplate reader at Ex/Em= 350/450 nm in a kinetic mode at 37°C for 30 – 60 min protected from light.

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
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

16. **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)