ab197006
Thrombin Activity Assay Kit (Fluorometric)

Instructions for Use

For the rapid, sensitive and accurate measurement of Thrombin activity in plasma.

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 5a, Last Updated 9 November 2023

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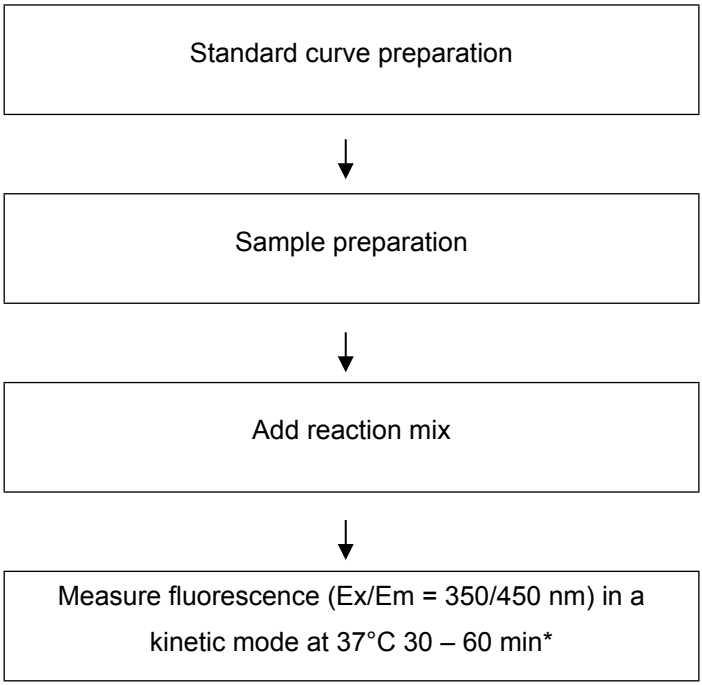
1. BACKGROUND

Thrombin Activity Assay Kit (fluorometric) (ab197006) utilizes the ability of thrombin to proteolytically cleave a synthetic substrate and release a fluorophore, AMC, which can be easily quantified by fluorescence reader. This assay kit is simple, rapid and can detect thrombin activity as low as 1 ng in samples.

Thrombin enzyme (Factor IIa), a serine protease, is an important clotting factor in the coagulation cascade that involves the conversion of soluble fibrinogen to insoluble active fibrin strands. In this pathway, prothrombin is proteolytically converted into an active thrombin. Thrombin is also a potent vasoconstrictor and mitogen implicated as a major factor in vasospasm following subarachnoid hemorrhage. Ruptured cerebral aneurysm bloodclots around a cerebral artery releases thrombin, which in turn induces acute and prolonged narrowing of the blood vessel, potentially resulting in cerebral ischemia and infarction (stroke). In addition, it is a pro-inflammatory enzyme that may influence the onset and progression of atherosclerosis.

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/Thrombin Enzyme Standard	5 µL	-20°C	-80°C
Thrombin Substrate	500 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 plate with clear flat bottom for fluorescent 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/Thrombin Enzyme Standard:**

Dilute 4 µL of standard in 12 µL of Thrombin dilution buffer to generate a 50 ng/µL standard stock solution. Mix. Aliquot standard so that you have enough volume to perform the desired number of tests. Avoid repeated freeze/thaw cycles. 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.

10. STANDARD PREPARATION

- Always prepare a fresh set of standards for every use.
- Diluted Thrombin Standard/thrombin enzyme standard solution can be stored at -80°C.

10.1 Prepare a 2.5 ng/µL standard by diluting 5 µL of the 50 ng/µL thrombin standard with 95 µL of thrombin dilution buffer.

10.2 Using 2.5 ng/µL thrombin standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard #	Volume of Standard (µL)	Assay Buffer (µL)	Final volume standard in well (µL)	End Conc. Thrombin in well
1	0	150	50	0 ng/well
2	6	144	50	5 ng/well
3	12	138	50	10 ng/well
4	18	132	50	15 ng/well
5	24	126	50	20 ng/well
6	30	120	50	25 ng/well

Each dilution has enough amount of standard to set up duplicate readings (2 x 50 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 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 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 (1/2 – 1/5 – 1/10).

11.2 Purified protein:

Purified protein 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.

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.**

12.1 **Set up Reaction wells:**

- Standard wells = 50 µL standard dilutions.
- Sample wells = 2 – 50 µL samples (adjust volume to 50 µL/well with Thrombin Assay Buffer).

12.2 **Reaction Mix:**

Prepare 50 µL of Reaction Mix for each reaction

Component	Reaction Mix (µL)
Thrombin Assay Buffer	**45**
Thrombin Substrate	**5**

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

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

12.3 Add 50 µL of Reaction Mix into each sample and standard well and mix.

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

NOTE: *Sample incubation time can vary depending on the Thrombin activity in samples. 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.*

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

13.4 Plot the corrected absorbance values for each standard as a function of the final concentration of thrombin.

13.5 Draw the best smooth curve through these points to construct the standard curve. Most plate reader software or Excel can plot these values and curve fit. Calculate the trendline equation based on your standard curve data (use the equation that provides the most accurate fit).

13.6 Extrapolate sample readings from the standard curve plotted using the following equation:

$$Time\ point\ value = \left(\frac{Corrected\ absorbance - (y - intercept)}{Slope}\right)$$

Time point = RFU1 or RFU2

13.7 Thrombin activity is calculated as:

$$\Delta RFU = RFU_2 - RFU_1$$

13.8 Use ΔRFU to obtain B ng thrombin.

13.9 Thrombin activity (in ng/mL or µg/L) in the test samples is calculated as:

$$Thrombin\ Activity = \left(\frac{B}{V}\right) * D$$

Where:

B = Amount of thrombin in the sample well (ng).

V = 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.

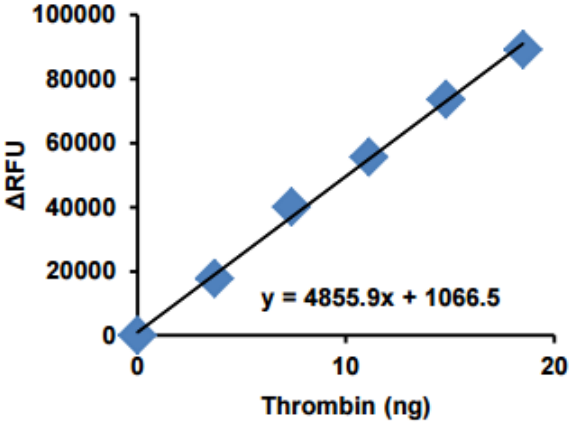


Figure 1. Typical thrombin standard calibration curve using fluorometric reading.

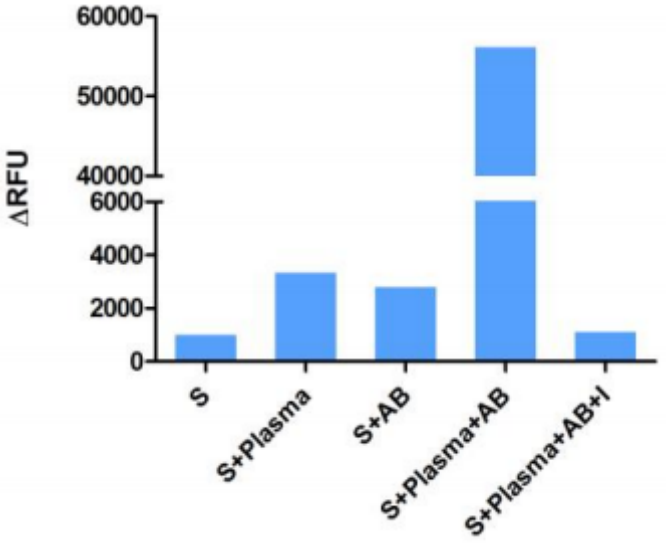


Figure 2: Thrombin activity was measured in plasma samples in the presence and absence of a thrombin inhibitor, PPACK Dihydrochloride (ab141451). S = Substrate, I = Inhibitor, AB = Activation Buffer containing Factor Xa.

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, substrate, dilution buffer and assay buffer (aliquot if necessary); get equipment ready.
- Prepare standard curve.
- Prepare samples in duplicate (find optimal dilutions to fit standard curve readings).
- Set up plate for standard (50 µL) and samples (50 µL).
- Prepare Thrombin Reaction Mix (Number samples + standards + 1).

Component	Fluorometric Reaction Mix (µL)
Thrombin Assay Buffer	**45**
Thrombin Substrate	**5**

- Add 50 µL of Thrombin Reaction Mix to the standard and sample wells.
- Incubate plate at 37°C 30 – 60 minutes protected from light, and read fluorescence at Ex/Em = 350/450 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. **FAQ**

18. INTERFERENCES

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

- RIPA: contains SDS which can destroy/decrease the activity of the enzyme.

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)