ab197006 –Thrombin Activity Assay (Fluorometric)

For rapid, sensitive and accurate measurement of Thrombin levels in plasma (citrate), plasma (EDTA), and purified protein.
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: http://www.abcam.com/ab197006 (use http://www.abcam.cn/ ab197006 for China, or http://www.abcam.co.jp/ab197006 for Japan)

Background:

Thrombin Activity Assay Kit (Fluorometric) (ab197006) provides a simple, rapid, and sensitive method for accurate quantification of analyte concentration in a variety of biological samples such as plasma (citrate), plasma (EDTA), and purified protein.

Thrombin Activity Assay utilizes the ability of thrombin to proteolytically cleave a synthetic peptide substrate and release AMC, a fluorophore, which can be quantified by fluorescence Ex/Em = 368/460 nm. This assay kit detects thrombin activity as low as 1 ng in samples.

Assay Summary:

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

Sample preparation

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Standard curve preparation

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Add reaction mix

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Measure fluorescence (Ex/Em = 368/460 nm) in kinetic mode for 60 min at 37°C.

QUICK ASSAY PROCEDURE

- Set plate reader to 37°C
- Thaw kit components and make aliquots to limit number of freeze/thaw cycles.
- Prepare samples in duplicate
- Prepare standard curve and Thrombin Positive Control
- Set up plate for standard (50 µL) and samples (50 µL).
- Prepare and add 50 µL Reaction Mix to each well.
- Measure fluorescence Ex/Em = 368/460 nm in kinetic mode for 60 minutes at 37°C. Alternatively for endpoint mode, incubate plate for 60 minutes at 37°C.

Precautions & Limitations:

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.
- 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.

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. Reconstituted components are stable for 2 months.

Materials Supplied:

Item	Quantity	Cap Code	Storage Temperature
Thrombin Assay Buffer	15 mL	Clear	-20°C
Thrombin Dilution Buffer	1 mL	Clear	-20°C
Thrombin Positive Control	5 µL	Green	**-80°C**
Thrombin Substrate	0.5 mL	Amber	-20°C
AMC Standard	100 µL	Amber	-20°C

Materials Required, Not Supplied:

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

- Microplate reader capable of measuring fluorescence at Ex/Em = 368/460 nm.
- 96 well black plate with flat bottom
- Microcentrifuge
- MilliQ water or other type of double distilled/deionized water (ddH_2O)

Reagent Preparation:

- Equilibrate reagents to room temperature before use.
- Briefly centrifuge small vials at low speed prior to opening.
- Aliquot reagents so that you have enough volume to perform the desired number of assays.
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Thrombin Assay Buffer: Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C.

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

Thrombin Positive Control: Dilute 4 µL of Thrombin Positive Control in 12 µL of Thrombin Dilution Buffer to generate a 50 ng/ µL standard stock solution. Make aliquots of the 50 ng/ µL solution to limit number of freeze/thaw cycles and store at -80°C. Further dilute to 2.5 ng/ µL by taking 2 µL of the 50 ng/ µL solution and adding to 38 µL of Thrombin Dilution Buffer. The 2.5 ng/ µL

Thrombin Positive Control solution will be the working material and should be discarded and made fresh after each use.

Thrombin Substrate: Ready to use as supplied. Equilibrate to room temperature before use. Store at -20°C and keep away from light exposure.

AMC Standard: Add 10 µL of AMC Standard to 90 µL of ddH_2O to generate a 100 µM AMC Standard solution. Do not store diluted Standard solution after use.

Sample Preparation:

- We recommend performing several dilutions or doses of your sample to ensure the readings are within the standard value range.

1. 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, snap freeze your samples in liquid nitrogen upon extraction and store them 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.
2. Interferences: Heparin-treated plasma is not recommended as a sample type. Heparin promotes antithrombin and will inhibit thrombin activity in samples. EDTA-treated plasma has been validated, but calculated Thrombin activity was reported lower than Citrate-treated plasma, which is the recommended sample.

Plasma Samples:
1. Collect whole blood into citrate or EDTA tubes. Keep sample at 4°C during preparation.
2. Remove cells by centrifuging sample for 10 minutes at 1,000 x g at 4°C.
3. Collect supernatant and transfer to a clean tube.
4. Keep on ice.

Recommended to dilute plasma 5:1 using Thrombin Assay Buffer.
Initial sample recommendation = 2-6 µL/well of diluted plasma

Standard Preparation:

- Always prepare a fresh set of standards for every use (Diluted standard solution should not be stored/reused).
- Each dilution has enough standard to set up duplicate readings (2 x 50 µL).

1. Add 0, 2, 4, 6, 8 and 10 µl of 100 µM AMC Standard into series of wells in a 96-well plate to generate 0, 200, 400, 600, 800, and 1000 pmol/well AMC. Adjust the volume to 50 µl/well with Thrombin Assay Buffer or prepare the standard curve dilution as described in the table below in a microplate or microcentrifuge tubes (sufficient for duplicate standard curves).

Standard #	Volume of 0.1 mM Standard (µL)	Thrombin Assay Buffer (µL)	Final volume standard in well (µL)	End AMC Amount (pmol/well)
1	0	125	50	0
2	5	120	50	200
3	10	115	50	400
4	15	110	50	600
5	20	105	50	800
6	25	100	50	1000

Assay Procedure:

- Keep enzymes and heat labile components and samples on ice during the assay.
- Equilibrate all other materials and reagents to room temperature prior to use.
- We recommend that you assay all standards, controls, and samples in duplicate.

1. Set up Reaction wells:
   - Standard wells = 50 µL standard dilutions.
   - Background Sample wells = 50 µL Thrombin Assay Buffer
   - Positive Control wells = 2 µL of 2.5 ng/ µL Thrombin Positive Control solution and adjust total well volume to 50 µL with Thrombin Assay Buffer (generates 5 ng Thrombin/well).
   - 2 –6 µL diluted samples (adjust volume to 50 µL/well with Thrombin Assay Buffer.
2. Each well (standards, positive controls, and samples) requires 50 µL of Reaction Mix as shown in the table below. To ensure consistency, use the table below to prepare a bulk mix for your assay using the following calculation:
   X µL component x (Number reactions +1).

Component	Volume (µL)
Thrombin Assay Buffer	45
Thrombin Substrate	5

3. Mix bulk reaction mix by inversion. Add 50 µL of the bulk reaction mix to each well (Sample, Standard, and Background Control).
4. Immediately measure fluorescence Ex/Em = 368/460 nm every 1 minute in kinetic mode for 60 minutes at 37°C. Alternatively for endpoint readout, incubate plate at 37°C for 60 minutes prior to measuring fluorescence.

Calculations:

1. Average the duplicate reading for each standard, control and sample.
2. Subtract the mean fluorescence (RFU) value of the blank (Standard #1, 0 pmoles/well) from all standard readings. This is the corrected fluorescence.
3. Plot the corrected fluorescence values for each standard as a function of the final amount of AMC (in pmoles/well).
4. Using a linear regression function, calculate the best fit curve through these points to construct the standard curve and determine the slope based on your standard curve data.

5. Subtract the average paired background RFU from the corresponding sample RFU. This is the corrected sample RFU.
6. Find two points within the linear range of each sample ($T_1$and T_2). Determine the ΔRFU those occurs between those two time points.
7. Extrapolate sample readings from the standard curve plotted using the following equation to determine the amount substrate metabolized in pmoles (*B*):

$$B = \left(\frac{Corrected\ RFU - (y - intercept)}{Slope}\right)$$

8. Activity of Thrombin (µU/mL) in the test samples is calculated as:

$$Sample\ Thrombin\ Activity = \frac{B}{V \times \Delta T} \times D = pmoles/min/ml\ = \mu U/mL$$

Where:
B is the amount of AMC produced, calculated from the standard curve (in pmoles)
Δ*T* is the linear phase reaction time $t_2 - t_1$ (in minutes, Δ*T* = 60 for an endpoint assay)
V is the amount of sample added to the well (in ml of fluid)
D is the sample dilution factor (if applicable, *D*=1 for undiluted samples)

Appendix:

Abcam's Thrombin Activity Assay Kit has been updated to include a standard curve that is based upon the amount of fluorophore released following cleavage of the thrombin peptide substrate, as opposed to a standard curve based upon nanograms of thrombin added per well. This is more consistent with the traditional definition of enzymatic activity, which is typically defined as one unit of enzyme activity is equal to one micromole of substrate metabolized per minute. A standard curve that is based upon mass of thrombin per well—as described in the calculation below—will likely have greater inter-assay variability than a standard that is based upon pmoles/well of fluorophore, hence we recommend using the protocol and calculations described above. However, for customers who would prefer to continue running this assay with the legacy protocol, including the standard curve based upon ng/well of thrombin, the historical standard curve setup is given in the instructions below:

Standard Curve Preparation:

1. Dilute the Thrombin Positive Control to 2.5 ng/µL by adding 38 µL of Thrombin Dilution Buffer to 2 µL of the 50 ng/ µL Thrombin Positive Control stock and mix well.
2. Add 0, 2, 4, 6, 8, and 10 µL of diluted Thrombin Positive Control into a series of wells in a 96-well plate.
3. Adjust the volume to 50 µL with Thrombin Assay Buffer to prepare 0, 5, 10, 15, 20, and 25 ng/well of Thrombin Positive Control.

Calculations:

1. Subtract the 0 standard reading from all readings and plot the Thrombin Standard Curve. Apply sample's ΔRFU to Thrombin Standard Curve to obtain corresponding Thrombin (B, in ng) and calculate the activity of Thrombin in the sample as:

$$Sample\ Thrombin\ Activity = \frac{B}{V}\ X\ Dilution\ Factor = \frac{ng}{ml} = \frac{\mu g}{L}$$

Where B is Thrombin amount from standard curve (ng)
V is sample volume added into the reaction well (ml)

Technical Hints

For additional helpful hints and tips on using our assay kits please visit:
https://www.abcam.com/en-us/support/product-support

Technical Support

For technical or commercial enquiries please go to:

www.abcam.com/contactus
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