Thrombin Activity Fluorometric Assay Kit

(Catalog # BN00635, 100 assays, Store kit at -20°C)

I. Introduction:

Thrombin enzyme (Factor IIa, EC 3.4.21.5), 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 blood clots 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. Assay Genie's Thrombin activity assay kit 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 Substrate-AMC $\xrightarrow{\text{Thrombin}}$ Cleaved substrate + AMC (Fluorescence)

II. Applications:

- Determine activity of pure Thrombin
- Detect the activity of Thrombin in plasma

III. Kit Contents:

Components	BN00635	Cap Code	Part Number
Thrombin Dilution Buffer	1 ml	Clear	BN00635-1
Thrombin Assay Buffer	15 ml	WM	BN00635-2
Thrombin Enzyme Standard	5 µl	Green	BN00635-3
Thrombin Substrate	0.5 ml	Red	BN00635-4

IV. User Supplied Reagents and Equipment:

- 96-well microplate with flat bottom. White plate is preferred for this assay.
- Fluorometer.

V. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge small vials at low speed prior to opening. Read entire protocol before performing the assay.

- **Thrombin Assay Buffer:** Bring to room temperature before use.
- **Thrombin Enzyme Standard:** Prepare a stock solution of Thrombin Enzyme (50 ng/µl) by adding 12 µl of Thrombin Dilution buffer to 4 µl of Thrombin Enzyme Standard. Mix. Aliquot & store at -80°C. Avoid repeated freeze/thaw.

VI. Thrombin Activity Assay Protocol:

1. **Sample Preparation:** Add 2-50 µl of sample containing Thrombin per well of 96-well plate and adjust the volume to 50 µl with Thrombin Assay Buffer.
2. **Standard Curve Preparation**: Dilute Thrombin Enzyme Standard to 2.5 ng/µl by adding 38 µl of Thrombin Dilution Buffer to 2 µl of Thrombin Enzyme stock solution (50 ng/µl). Mix and add 0, 2, 4, 6, 8 and 10 µl of diluted Thrombin Enzyme Standard (2.5 ng/µl) into a series of wells in a 96-well plate. Adjust the volume to 50 µl with Thrombin Assay Buffer to prepare 0, 5, 10, 15, 20 and 25 ng/well of Thrombin Enzyme Standard.

   Note: Store the diluted Thrombin Enzyme Standard solution at -80°C.
3. **Substrate Mix:** Prepare enough reagents for the number of assays to be performed. Prepare 50 µl of Substrate Mix for Standard & sample wells.

Thrombin Assay Buffer	45 µl
Thrombin Substrate	5 µl

   Mix and add 50 µl of Thrombin Substrate Mix into Standard and sample well(s). Mix well.
4. **Measurement:** Measure fluorescence in kinetic mode for 30-60 min. at 37°C (Ex/Em = 350/450 nm). Choose two time points (T_1 & T_2) in the linear range of the plot and obtain the corresponding values for the fluorescence (RFU1 and RFU2).
5. **Calculations**: Subtract 0 Standard reading from all readings. 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:

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

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

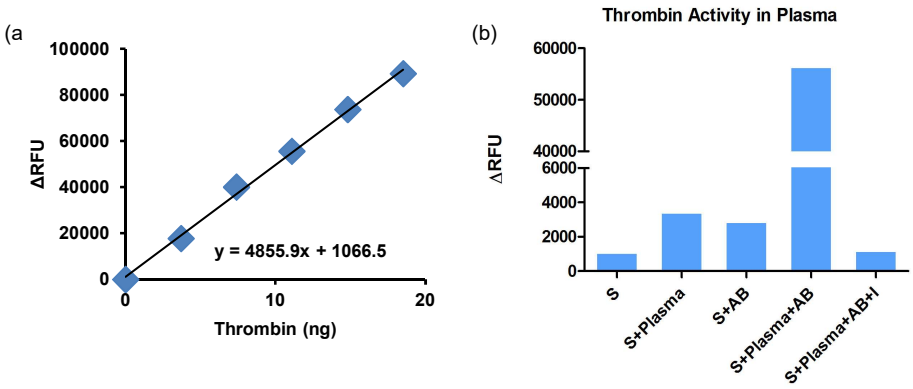

Figure: (a) Standard plot of Thrombin activity, (b) Thrombin activity was measured in plasma samples in the presence and absence of a Thrombin inhibitor, PPACK Dihydrochloride. S = Substrate, I = Inhibitor, AB = Activation Buffer containing Factor Xa. Assays were performed following the kit protocol.

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