Thrombin Inhibitor Screening Kit (Fluorometric) (#BN00636)

(Catalog # BN00636; 100 assays, Store kit at -20°C)

I. Introduction:

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 (EC 3.4.21.5) 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. Assay Genie's Thrombin Inhibitor Screening Kit 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 Substrate-AMC $\xrightarrow{\text{Thrombin}}$ Cleaved substrate + AMC (Fluorescence)

Thrombin Substrate-AMC $\xrightarrow{\text{Thrombin + Inhibitor}}$ Decrease in fluorescence/No fluorescence

II. Applications:

- Screening potential inhibitors of thrombin
- Characterizing/studying thrombin inhibitors in plasma samples

III. Kit Contents:

Components	BN00636	Cap Code	Part Number
Thrombin Dilution Buffer	1 ml	Clear	BN00636-1
Thrombin Assay Buffer	15 ml	WM	BN00636-2
Thrombin Enzyme	5 µl	Green	BN00636-3
Thrombin Substrate	0.5 ml	Red	BN00636-4
Thrombin Inhibitor (PPACK Dihydrochloride, 2 mM)	10 µl	Blue	BN00636-5

IV. User Supplied Reagents and Equipment:

- 96-well plate with flat bottom. White plate is preferred for this assay.
- Multi-well spectrophotometer

V. Storage Condition 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:** Add 215 µl of Thrombin Dilution Buffer to prepare stock solution. Mix well by pipetting up and down. Aliquot & store at -80°C. Avoid repeated freeze/thaw.

VI. Thrombin Inhibitor Screening Protocol:

1. **Enzyme Solution Preparation**: Mix enough reagents for the number of assays to be performed. For each well, prepare 50 µl of Thrombin enzyme solution.

Thrombin Assay Buffer	48 µl
Thrombin Enzyme stock solution	2 µl

   Mix & add 50 µl of Thrombin Enzyme Solution into desired wells.

2. **Screening compounds, Inhibitor Control & Enzyme Control Preparations:** Dissolve candidate inhibitors into proper solvent. Dilute to 10X the desired test concentration with Thrombin Assay Buffer. Add 10 µl diluted test inhibitors (Sample, S) or Thrombin Assay Buffer into Thrombin Enzyme containing wells (Enzyme Control, EC). As an Inhibitor Control (IC), add 1 µl Thrombin Inhibitor and 9 µl Thrombin Assay Buffer to Thrombin Enzyme well(s). Incubate at room temperature for 10-15 min.

3. **Substrate Preparation:** For each well, prepare 40 µl of substrate solution.

Thrombin Assay Buffer	35 µl
Thrombin Substrate	5 µl

   Mix & add 40 µl of Thrombin Substrate solution into each well. Mix well.

4. **Measurement:** Measure fluorescence in a 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). Irreversible inhibitors that inhibit the Thrombin activity completely at the tested concentration will have ΔRFU = 0 and will show 100% Relative Inhibition.

5. **Calculations**: Calculate the slope for all Samples (S), including Enzyme Control (EC), by dividing the net ΔRFU (RFU_2-RFU_1) values with the time ΔT (T_2-T_1).

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

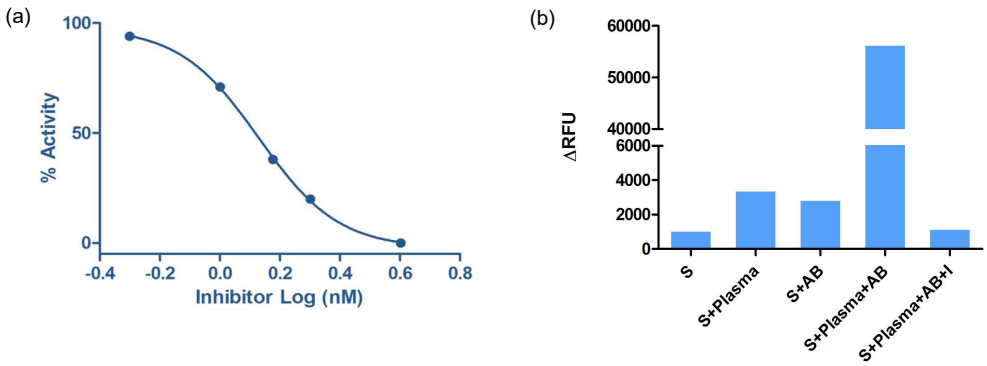

Figure: (a) Inhibition of thrombin activity by a thrombin Inhibitor (PPACK Dihydrochloride). (b) Thrombin activity was measured in plasma samples in the presence and absence of Thrombin Inhibitor (PPACK Dihydrochloride). S = Substrate, I = Inhibitor, AB = Activation Buffer containing Factor Xa. Assays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

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