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Product Information

Thrombin Inhibitor Screening Kit (Fluorometric)

Catalog Number **MAK243**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Thrombin (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 use while others are in clinical development.

This 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 (λ_{ex} = 350 nm/ λ_{em} = 450 nm). In the presence of thrombin specific inhibitors, the 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.

Components

The kit is sufficient for 100 assays in 96 well plates.

Thrombin Dilution Buffer Catalog Number MAK243A	1 mL
Thrombin Assay Buffer Catalog Number MAK243B	15 mL
Thrombin Enzyme Catalog Number MAK243C	5 µL
Thrombin Substrate Catalog Number MAK243D	0.5 mL
Thrombin Inhibitor (PPACK Dihydrochloride, 2 mM) Catalog Number MAK243E	10 µL

Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – white plates are preferred for this assay.
- Fluorescence multiwell plate reader

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Briefly centrifuge small vials at low speed prior to opening.

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 and store at –20 °C. Avoid repeated freeze/thaw cycles.

Storage/Stability

Store the kit at –20 °C, protected from light. Briefly centrifuge small vials prior to opening.

Procedure
Read entire protocol before performing the assay.

Enzyme Solution Preparation
Mix enough Enzyme Solution for the number of assays to be performed. For each well, prepare 50 µL of Thrombin Enzyme Solution.

Table 1.
Preparation of Thrombin Enzyme Solution

Reagents	Volume
Thrombin Assay Buffer	48 µL
Thrombin Enzyme stock solution	2 µL

Mix and add 50 µL of Thrombin Enzyme Solution into desired wells.

Screening compounds, Inhibitor Control, and Enzyme Control Preparations
Dissolve candidate inhibitors to be tested into proper solvent. Dilute to 10× the desired test concentration with Thrombin Assay Buffer. Add 10 µL of diluted test inhibitor (Sample, S) or Thrombin Assay Buffer (Enzyme Control, EC) into wells containing Thrombin Enzyme Solution. As an Inhibitor Control (IC), add 1 µL of Thrombin Inhibitor (Catalog Number MAK243E) and 9 µL of Thrombin Assay Buffer to Thrombin Enzyme well(s). Incubate at room temperature for 10–15 minutes.

Substrate Preparation: For each well, prepare 40 µL of substrate solution, see Table 2.

Table 2.
Preparation of Substrate Solution

Reagents	Volume
Thrombin Assay Buffer	35 µL
Thrombin Substrate	5 µL

Mix and add 40 µL of Thrombin Substrate Solution into each well. Mix well.

Measurement
Measure fluorescence in kinetic mode for 30–60 minutes at 37 °C (λ_{ex} = 350 nm/λ_{em} = 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 (RFU_1 and RFU_2). Irreversible inhibitors that inhibit the FXa activity completely at the tested concentration will have ΔRFU = 0 and will show 100% Relative Inhibition.

Results
Calculations
Calculate the slope for all Samples (S), including Enzyme Control (EC), by dividing the net ΔRFU (RFU_2–RFU_1) values by the time ΔT (T_2–T_1).

% Relative Inhibition = [Slope(EC)–Slope(S)]/Slope(EC) × 100

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay Not Working	Cold assay buffer	Assay Buffer must be at room temperature
	Omission of step in procedure	Refer and follow Technical Bulletin precisely
	Plate reader at incorrect wavelength	Check filter settings of instrument
	Type of 96 well plate used	White plates are preferred for this assay.
Samples with erratic readings	Samples prepared in different buffer	Use the Assay Buffer provided or refer to Technical Bulletin for instructions
	Cell/Tissue culture samples were incompletely homogenized	Repeat the sample homogenization, increasing the length and extent of homogenization step.
	Samples used after multiple freeze-thaw cycles	Aliquot and freeze samples if samples will be used multiple times
	Presence of interfering substance in the sample	If possible, dilute sample further
	Use of old or inappropriately stored samples	Use fresh samples and store correctly until use
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Use of expired kit or improperly stored reagents	Check the expiration date and store the components appropriately
	Allowing the reagents to sit for extended times on ice	Prepare fresh Reaction Mix before each use
	Incorrect incubation times or temperatures	Refer to Technical Bulletin and verify correct incubation times and temperatures
	Incorrect volumes used	Use calibrated pipettes and aliquot correctly
Non-linear standard curve	Use of partially thawed components	Thaw and resuspend all components before preparing the reaction mix
	Pipetting errors in preparation of standards	Avoid pipetting small volumes
	Pipetting errors in the Reaction Mix	Prepare a Reaction Mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the plate well
	Standard stock is at incorrect concentration	Refer to the standard dilution instructions in the Technical Bulletin
	Calculation errors	Recheck calculations after referring to Technical Bulletin
	Substituting reagents from older kits/lots	Use fresh components from the same kit
Unanticipated results	Samples measured at incorrect wavelength	Check the equipment and filter settings
	Samples contain interfering substances	If possible, dilute sample further
	Sample readings above/below the linear range	Concentrate or dilute samples so readings are in the linear range

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