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Product Information

N′-Nicotinamide Methyltransferase (NNMT) Inhibitor Screening Kit

Catalog Number **MAK299**
Storage Temperature –70 °C

TECHNICAL BULLETIN

Product Description

N′-Nicotinamide Methyltransferase (E.C. 2.1.1.1, NNMT) catalyzes the N-methylation of nicotinamide, pyridines, and other analogues using S-adenosyl methionine (SAM) as the donor resulting in the production of 1-methylnicotinamide (MNA). NNMT plays a significant role in the regulation of metabolic pathways and is expressed at markedly high levels in several kinds of cancers, neurodegenerative diseases, obesity, and diabetes, indicating it is a potential molecular target for therapy.

This NNMT inhibitor screening kit utilizes SAM as the methyl group donor and nicotinamide as the substrate. NNMT methylates nicotinamide generating S-adenosyl-homocysteine (SAH) and 1-methylnicotinamide. The SAH is hydrolyzed by SAH hydrolase to form homocysteine, the free thiol group of which is detected using the Thiol Detecting Probe, generating an enhanced fluorescence signal (λ_{ex} = 392 nm/ λ_{em} = 482 nm). In the presence of an NNMT inhibitor, the enzymatic activity is inhibited resulting in decreased fluorescence. This assay kit is a simple, sensitive, and rapid tool to screen potential inhibitors of NNMT.

Components

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

NNMT Assay Buffer Catalog Number MAK299A	22 mL
NNMT Enzyme Catalog Number MAK299B	50 µL
S-Adenosylmethionine (SAM) Catalog Number MAK299C	4 vials
Nicotinamide Catalog Number MAK299D	3 × 1.5 mL
Enzyme-I Catalog Number MAK299E	200 µL
Enzyme-II Catalog Number MAK299F	1 vial
1-Methylnicotinamide (MNA) (150 mM) Catalog Number MAK299G	20 µL
Thiol Detecting Probe (DMSO) Catalog Number MAK299H	200 µL
SAM Reconstitution Buffer Catalog Number MAK299I	500 µL

Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – black plates are preferred for this assay
- Fluorescence multiwell plate reader
- Isopropyl alcohol chilled to –20 °C
- Dimethylsulfoxide (DMSO)

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 prior to opening.

NNMT Assay Buffer - Warm to 37°C before use.

NNMT Enzyme – Aliquot after the first thaw and store at –70 °C. Stable at –70 °C for two months. Avoid repeated freeze/thaw cycles. Keep on ice while in use.

Nicotinamide - Store at –70 °C. Use within two months.

Enzyme-I – Aliquot after the first thaw and store at –70 °C. Stable at –70 °C for two months. Avoid repeated freeze/thaw cycles. Keep on ice while in use.

S-Adenosylmethionine (SAM) (lyophilized) – Reconstitute each vial with 55 µL of SAM Reconstitution Buffer as needed. Pipette up and down to dissolve completely. Store at –70 °C. Avoid repeated freeze/thaw cycles. Use reconstituted SAM within two weeks. Keep on ice while in use. Lyophilized product is stable at –70 °C for two months.

Enzyme-II (Lyophilized) – Reconstitute with 220 µL of NNMT Assay Buffer. Aliquot and store at –70 °C. Stable for two months at –70 °C.

1-Methylnicotinamide (MNA) – Store at –70 °C. Avoid repeated freeze/thaw cycles. Use within two months.

Thiol Detecting Probe – Store at –20 °C or –70 °C. Thaw and mix well before use.

SAM Reconstitution Buffer – Store at –20 °C or –70 °C.

Storage/Stability
Store kit at –70 °C, protected from light. Briefly centrifuge small vials prior to opening.

Procedure
Read entire protocol before performing the assay. Any deviations can result in sub-optimal results.

Screening Compounds, Inhibitor Control, and Blank Control Preparation
Dissolve test inhibitors in an appropriate solvent to make a 3-100× stock solution. Dilute the highest desired test concentration to 3× with NNMT Assay Buffer. Prepare the reactions as shown in the table. If desired, serial dilutions of test inhibitors may be performed at this time, to a final volume of 50 µL.

Table 1.
Preparation of Screening Compounds, Inhibitor Control, and Blank Control

Reagent	Sample	Enzyme Control	Background Control*	Inhibitor Control
Test Inhibitor (3×)	50 µL	–	–	–
NNMT Assay Buffer	–	50 µL	75 µL	48 µL
Inhibitor Control (MNA)	–	–	–	2 µL

Notes: If desired, include a Solvent Control to test the effect of the solvent on enzyme activity. NNMT is sensitive to as low as 0.2 % DMSO in the assay.

The Thiol Detecting Probe will react with thiol groups on the enzymes used in the assay, hence a Background Control (BC) containing the reaction mix only without any Nicotinamide is necessary.

NNMT Reaction Mix
Dilute NNMT Enzyme and Enzyme-I 1:5 in NNMT Assay Buffer. Prepare a 75 µL Reaction Mix for each well (Sample, Enzyme Control, Background Control, and Inhibitor Control), see Table 2.

Table 2.
Preparation of NNMT Reaction Mix

Reagent	Volume
NNMT Assay Buffer	58.5 µL
1:5 diluted NNMT Enzyme	2.5 µL
1:5 diluted Enzyme-I	10 µL
SAM	2 µL
Enzyme II	2 µL

Mix and add 75 µL/well. Mix well**.

** Note: Mix the contents in the wells thoroughly using a multichannel pipette.

NNMT Assay
To all wells, except the Background Control, add 25 µL of Nicotinamide using a multichannel pipette. Mix well** and incubate at 37 °C for 15 minutes. Stop the reaction by adding 50 µL of chilled isopropyl alcohol (not provided) into each well, mix,** and keep on ice for 5 minutes. For each well, prepare 50 µL of Thiol Detecting Probe working solution by adding 2 µL of Thiol Detecting Probe into 48 µL of DMSO (not provided) just before use. Add 50 µL of Thiol Detecting Probe working solution into each well. Mix** and incubate at room temperature for 5 minutes and read immediately.

Measure fluorescence (λ_{ex} = 392 nm/λ_{em} = 482 nm)

Results

Calculations

Subtract the Background Control reading from all (Sample, Enzyme Control and Inhibitor Control) readings to obtain ΔRFU for each.

Set the ΔRFU of Enzyme Control [EC] as 100%, and calculate % Inhibition or % Relative Activity of the test inhibitors as follows:

$$\% \text{ Relative Inhibition} = \frac{(\Delta\text{RFU of EC} - \Delta\text{RFU of S})}{\Delta\text{RFU of EC}} \times 100$$

$$\% \text{ Relative Activity} = \frac{\Delta\text{RFU of S}}{\Delta\text{RFU of EC}} \times 100$$

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	Black plates are recommended 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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