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Product Information

Fluorometric Sphingomyelin Assay Kit

Catalog Number **MAK154**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Sphingomyelin, a sphingolipid consisting of a phospholipid (typically phosphocholine) attached to a ceramide moiety, is a major component of cellular membranes. Sphingomyelin is enriched in the exoplasmic leaflet of the cell membrane. Within the cell membrane, sphingomyelin interacts with cholesterol to form lipid rafts. The metabolism of sphingomyelin contributes to cellular signaling via the release of ceramide, a second messenger that participates in multiple cellular signaling pathways. Sphingomyelin accumulates in Niemann-Pick disease due to deficiencies in sphingomyelinase activity.

The Sphingomyelin Assay Kit provides a simple and direct procedure for measuring sphingomyelin in a variety of samples such as blood or cell extracts. Sphingomyelin is determined by a coupled enzyme assay, in which the hydrolysis of sphingomyelin by sphingomyelinase produces phosphocholine, which results in a fluorescent product (λ_{ex} =540/λ_{em} = 590 nm) proportional to the sphingomyelin present.

Components

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

Enzyme Mix 1 ea
Catalog Number MAK154A

Sphingomyelinase 1 vl
Catalog Number MAK154B

Red Detection Reagent 1 vl
Catalog Number MAK154C

SMase Reaction Buffer 20 mL
Catalog Number MAK154D

Assay Buffer 5 mL
Catalog Number MAK154E

Sphingomyelin, 50 mM in 20 µL 1 vl
Catalog Number MAK154F

DMSO 0.2 mL
Catalog Number MAK154G

Reagents and Equipment Required but Not Provided.

- 96 well flat-bottom plate – It is recommended to use black plates with clear bottoms for fluorometric assays.
- Fluorescence multiwell plate reader
- Bovine Serum Albumin (Catalog Number A4737 or equivalent)
- Phosphate Buffered Saline (Catalog Number 79378 or equivalent)

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 vials before opening. To maintain reagent integrity, avoid repeated freeze/thaw cycles. Allow all reagents to come to room temperature before use.

100× Sphingomyelinase (SMase) Working Stock – Reconstitute Sphingomyelinase vial with 50 µL of a PBS solution containing 0.1% BSA. Mix well by pipetting (do not vortex), then aliquot and store, protected from light, at –20 °C.

Red Detection Reagent – Reconstitute with 80 µL of DMSO to make a 200× Red Detection Reagent Stock Solution. Mix well by pipetting (do not vortex), then aliquot and store, protected from light, at –20 °C.

Storage/Stability

The kit is shipped on dry ice and storage at –20 °C, protected from light, is recommended.

Procedure
All samples and standards should be run in duplicate.

Sphingomyelin Standards
Add 2 μL of the 50 mM Sphingomyelin to 1,000 μL of SMase Reaction Buffer to prepare a 100 μM Sphingomyelin Standard Solution. Take 200 μL of the 100 μM Standard Solution and prepare 3-fold serial dilutions with SMase Reaction Buffer generating 100, 33, 11, 3.3, 1.1, 0.36, 0.122, and 0 (blank) μM standards. Add 50 μL of the serially diluted standards into a 96 well plate.

Sample Preparation
Add up to 50 μL of sample to wells. Bring samples to a final volume of 50 μL with SMase Reaction Buffer. The Red Detection Reagent is not stable in the presence of thiols (such as DTT and 2-mercaptoethanol) or at high pH (>8.5). The final concentration of thiol in the reaction mix should be <10 μM. The reaction should be performed at pH 7–8.

Note: For unknown samples, it is suggested to test several sample dilutions to ensure the readings are within the linear range of the standard curve.

Assay Reaction
1. Set up the 1× Sphingomyelinase Working Solution according to the scheme in Table 1. The Sphingomyelin Working Solution is enough for one plate, as 50 μL of the working solution is required for each reaction (well).
   Note: The Sphingomyelin Working Solution is not stable and should be used promptly.

Table 1.
Sphingomyelin Working Solution

Reagent	Volume
100× SMase Working Stock	50 μL
SMase Reaction Buffer	5 mL

2. Add 50 μL of the Sphingomyelin Working Solution to each of the sample, blank, and standard wells. Mix well using a horizontal shaker or by pipetting, and incubate the reaction for 1–2 hours at 37 °C. Cover the plate during the incubation.
3. Add 5 mL of Assay Buffer into 1 bottle of Enzyme Mix and mix well. Add 25 μL of the 200× Red Detection Reagent stock solution to the reconstituted Enzyme Mix solution to make the Sphingomyelin Assay Mixture.
   Note: The Sphingomyelinase Assay Mixture should be protected from light and used promptly. Storage results in high assay background. The Sphingomyelinase Assay Mixture may appear cloudy. This is normal and will not interfere with assay performance.
4. Add 50 μL of the Sphingomyelin Assay Mixture into each of the sample, blank, and standard wells for a total assay volume of 150 μL/well.
5. Incubate the plate for 1–2 hours at room temperature. Protect the plate from light during the incubation.
6. Measure the fluorescence intensity (λ_{ex} =540/ λ_{em} = 590 nm (cut off at 570 nm).

Note: This assay can be adapted for use with 384 well plates. When working with 384 well plates, add 25 μL of standard, sample, working solution, enzyme mix solution, and assay mixture to each well at the respective steps.

Results
Calculations
The background blank for the assay is the value obtained for the 0 (blank) Sphingomyelin Standard. Correct for the background by subtracting the blank value from all readings. Background values can be significant and must be subtracted from all readings.

Use the values obtained from the standards to plot a standard curve.
Note: A new standard curve must be set up each time the assay is run.

The concentration of sphingomyelin present in the samples may be determined from the standard curve.

Troubleshooting Guide

Problem	Possible Cause	Suggested Solution
Assay not working	Cold Reagents	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	For fluorometric assays, use black plates with clear bottoms
Samples with erratic readings	Samples prepared in an incompatible buffer	Make sure the assay buffer has a pH between 7–8 and that thiols are present at less than 10 μM.
	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 needed to use 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 Master 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
	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
	Samples measured at incorrect wavelength	Check the equipment and filter settings
Unanticipated results	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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