

**Sphingomyelinase Inhibitor
Screening Assay Kit**

Item No. 700330

TABLE OF CONTENTS

GENERAL INFORMATION	3	Materials Supplied
	4	Precautions
	4	If You Have Problems
	4	Storage and Stability
	4	Materials Needed but Not Supplied
INTRODUCTION	5	Background
	5	About This Assay
PRE-ASSAY PREPARATION	7	Reagent Preparation
ASSAY PROTOCOL	8	Plate Set Up
	10	Performing the Assay
ANALYSIS	12	Calculations
	14	Performance Characteristics
RESOURCES	15	Troubleshooting
	16	References
	17	Related Products
	18	Warranty and Limitation of Remedy
	19	Plate Template
	20	Notes

GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity/Size
700221	SMase Assay Buffer (5X)	1 vial/10 ml
700331	SMase Sphingomyelin Conjugate	2 vials/600 µl
700332	Sphingomyelinase Inhibitor Screening Enzyme	2 vials
700333	SMase Detector	1 vial/100 µl
400013	96-Well Solid Plate (white)	1 plate
400012	96-Well Cover Sheet	1 cover

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 975-3999. We cannot accept any returns without prior authorization.



WARNING: This product is for laboratory research use only; not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

E-Mail: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorometer with the capacity to measure fluorescence using an excitation wavelength of 375-385 nm and an emission wavelength of 510-520 nm
2. Adjustable pipettes and a repeat pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Sphingomyelinase (SMase) is a hydrolase enzyme that is involved in sphingolipid metabolism. SMase is a member of the DNase I superfamily of enzymes and is responsible for the breakdown of sphingomyelin into phosphocholine and ceramide. The activation of SMase has been suggested as a major route for the production of ceramide in response to cellular stress.¹ SMases are important in many physiological and pathophysiological processes, including: 1. lysosomal digestion of sphingomyelin, which is important for normal neuronal and vascular function; 2. ceramide-mediated signal transduction, leading to cytokine-induced apoptosis, cellular differentiation, and various immune and inflammatory responses; 3. lipoprotein aggregation within the vessel wall, which is a key event in atherogenesis; and 4. intracellular cholesterol trafficking and metabolism.²⁻⁵

Finding inhibitors to SMase (both acidic and neutral enzymes) could be beneficial in determining agents which reduce SMase activity and ceramide levels leading to attenuation of apoptosis and cellular proliferation, possible anti-depressant effects in depressive disorders, and beneficial clinical effects in acute or chronic neurodegenerative disorders, such as stroke and Alzheimer's dementia.⁶⁻⁸ Synthesis of SMase inhibitors could be beneficial in the investigation and establishment of new therapeutic concepts for several diseases by using SMase as an actual target for new drug design.⁹

About This Assay

Cayman's Sphingomyelinase Inhibitor Screening Assay provides a convenient method for screening sphingomyelinase inhibitors. This assay includes a neutral bacterial SMase. Even though bacterial SMase only shares a 20% sequence homology with mammalian neutral SMase, it has been shown to share a common catalytic site as the mammalian SMase and similar inhibition profile.^{10,11} Cleavage of the sphingomyelin conjugate by SMase results in the release of a ceramide analog containing a free thiol which is detected by the fluorescent SMase Detector (Figure 1, see page 6). This fluorescence is analyzed with an excitation wavelength of 375-385 nm and an emission wavelength of 510-520 nm.¹²

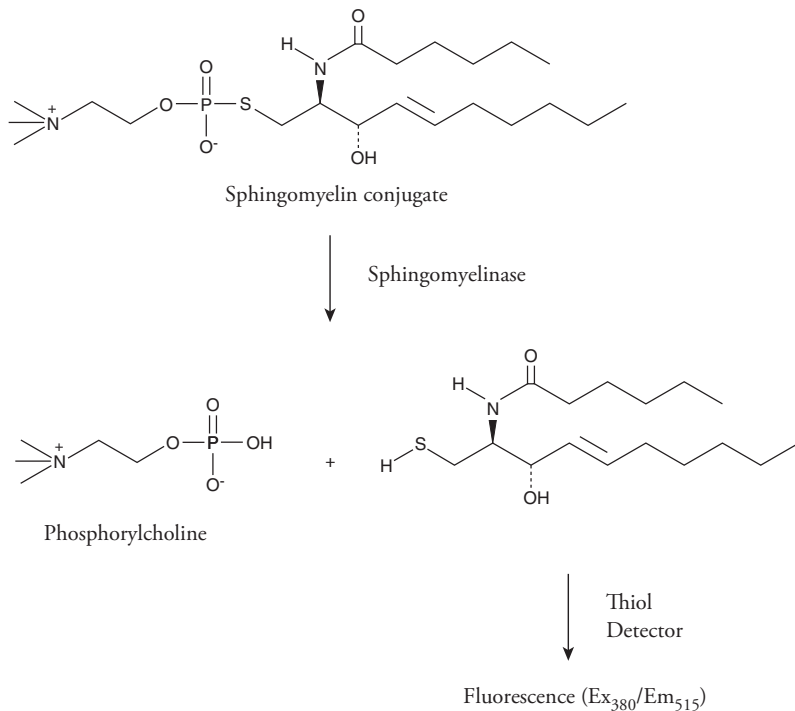


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. SMase Assay Buffer (5X) - (Item No. 700221)

The vial contains 10 ml of 500 mM Tris-HCl, pH 7.4, containing 50 mM MgCl₂. Thaw the Assay Buffer at room temperature. Dilute the contents of the vial with 40 ml of HPLC-grade water. This final 1X Assay Buffer (100 mM Tris-HCl, pH 7.4 containing 10 mM MgCl₂) should be used in the assay and for diluting reagents. When stored at 4°C, this diluted Assay Buffer is stable for at least six months.

2. SMase Sphingomyelin Conjugate - (Item No. 700331)

Each vial contains 600 µl of sphingomyelin conjugate in ethanol. The reagent is ready to use as supplied. One vial is enough conjugate to assay 60 wells. If additional wells are being utilized, then dilute the other vial of conjugate. Any unused conjugate should be stored at -20°C.

3. Sphingomyelinase Inhibitor Screening Enzyme - (Item No. 700332)

Each vial contains a lyophilized powder of neutral sphingomyelinase from *Bacillus cereus*. Dissolve the contents of one vial in 1 ml of 1X Assay Buffer. The reconstituted enzyme mixture is stable for two hours on ice.

Dilute 600 µl of the reconstituted SMase enzyme with 5.4 ml of 1X Assay Buffer and vortex. Place enzyme on ice. This is enough enzyme to assay 60 wells. If additional wells are being utilized, then dilute the other vial of enzyme. The diluted enzyme is stable for two hours.

4. SMase Detector - (Item No. 700333)

The vial contains 100 µl of thiol detector in dimethylsulfoxide (DMSO). Prior to use, dilute 25 µl of the SMase Detector with 1.625 ml of 1X Assay Buffer and vortex. *NOTE: Keep solution from exposure to light as much as possible.* The diluted detector solution is stable for 60 minutes. Any unused detector should be stored at -20°C.

Plate Set Up

There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% Initial Activity wells and three wells designated as Background wells. We suggest that each inhibitor be assayed in triplicate and that you record the contents of each well on the template sheet provided on page 19. A typical layout of samples and inhibitors to be measured in triplicate is shown in Figure 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	A	A	A	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW - Background Wells

A - 100% Initial Activity Wells

1-30 - Inhibitor Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 200 μ l in all wells
- Use the diluted Assay Buffer and the diluted solutions of the enzyme mixture and Detector in the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended that the samples be assayed at least in triplicate, but it is the user's discretion to do so.
- The assay is performed at 37°C.
- Monitor the fluorescence with an excitation wavelength of 375-385 nm and an emission wavelength of 510-520 nm.

Performing the Assay

1. **Background Wells** - Add 155 μl of 1X Assay Buffer, 10 μl of Sphingomyelin Conjugate, 25 μl of diluted SMase Detector Solution, and 10 μl of solvent (same solvent used to dissolve the inhibitor) to three wells (see **Sample Plate Format**, Figure 2, page 8).
2. **100% Initial Activity Wells** - Add 55 μl of 1X Assay Buffer, 10 μl of Sphingomyelin Conjugate, 25 μl of diluted SMase Detector Solution, and 10 μl of solvent (same solvent used to dissolve the inhibitor) to three wells.
3. **Inhibitor Wells** - Add 55 μl of 1X Assay Buffer, 10 μl of Sphingomyelin Conjugate, 25 μl of diluted SMase Detector Solution, and 10 μl of Inhibitor* to three wells.

Well	Assay Buffer	SMase Enzyme	Conjugate	Detector	Solvent	Inhibitor
Background	155 μl	-	10 μl	25 μl	10 μl	-
100% Initial Activity	55 μl	100 μl	10 μl	25 μl	10 μl	-
Inhibitor	55 μl	100 μl	10 μl	25 μl	-	10 μl

Table 1. Pipetting summary

*Inhibitors can be dissolved in methanol, DMSO, or ethanol and should be added to the assay in a final volume of 10 μl . In the event that an appropriate concentration of inhibitor is completely unknown, we recommend that several dilutions of the inhibitor be made.

4. Initiate the reactions by adding 100 μl of the diluted SMase Enzyme to the 100% Initial Activity and Inhibitor wells. Carefully shake the microtiter plate for 10 seconds to mix and cover with the plate cover. Incubate for 30 minutes at 37°C.
5. Remove the plate cover and read using an excitation wavelength of 375-385 nm and an emission wavelength of 510-520 nm.

NOTE: If a diluted inhibitor is colored, it is possible it will interfere by causing an increase in fluorescence. Correct for this interference, by adding the Inhibitor to some Background wells. The Background wells with inhibitor should be subtracted from the corresponding Inhibitor wells.

ANALYSIS

Calculations

1. Determine the average fluorescence of the Background, Initial Activity (IA), and Inhibitor wells.
2. Subtract the average fluorescence value of the Background wells from the average fluorescence value of the 100% Initial Activity and the Inhibitor wells.
3. Determine the % Inhibition or % Initial Activity for each sample.

$$\% \text{ Inhibition} = \left[\frac{\text{IA} - \text{Inhibitor}}{\text{IA}} \right] \times 100$$

$$\% \text{ Initial Activity} = \frac{\text{Inhibitor}}{\text{IA}} \times 100$$

4. Graph the percent Inhibition or percent Initial Activity as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition). The inhibition of sphingomyelinase by GW 4869 is shown in Figure 3, on page 13 as an example.

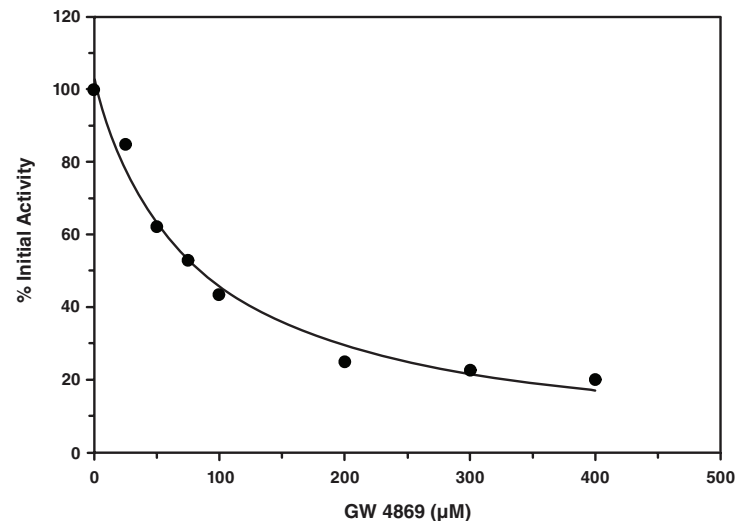


Figure 2. Inhibition of sphingomyelinase by GW 4869 ($IC_{50} = 82 \mu\text{M}$)

Performance Characteristics

Precision:

When a series of 48 sphingomyelinase measurements were performed on the same day, the intra-assay coefficient of variation was 6.8%. When a series of 16 sphingomyelinase measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 6.1%.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence was detected above background in the inhibitor wells	A. Enzyme or sphingomyelin conjugate was not added to the well(s) B. Inhibitor concentration is too high and inhibited all of the enzyme activity	A. Make sure to add all of the components to the wells B. Reduce the concentration of the inhibitor and re-assay
Fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read
No inhibition was seen with inhibitor	A. The inhibitor concentration is not high enough B. The inhibitor is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay

References

1. Hannun, Y.A. and Obeid, L.M. The ceramide-centric universe of lipid-mediated cell regulation: Stress encounters of the lipid kind. *J. Biol. Chem.* **277**(29), 25847-25850 (2002).
2. Kolesnick, R.N. Sphingomyelin and derivatives as cellular signals. *Prog. Lipid Res.* **30**(1), 1-38 (1991).
3. Tabas, I., Li, Y., Brocia, R.W., *et al.* Lipoprotein lipase and sphingomyelinase synergistically enhance the association of atherogenic lipoproteins with smooth muscle cells and extracellular matrix: A possible mechanism for low density lipoprotein and lipoprotein(a) retention and macrophage foam cell formation. *J. Biol. Chem.* **268**(27), 20419-20432 (1993).
4. Pörn, M.I. and Slotte, J.P. Localization of cholesterol in sphingomyelinase-treated fibroblasts. *Biochem. J.* **308**, 269-274 (1995).
5. Smith, E.L. and Schuchman, E.H. The unexpected role of acid sphingomyelinase in cell death and the pathophysiology of common diseases. *FASEB J.* **22**, 3419-3431 (2008).
6. Gulbins, E. and Li, P.L. Physiological and pathophysiological aspects of ceramide. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **290**, R11-R26 (2006).
7. Kolesnick, R. The therapeutic potential of modulating the ceramide/sphingomyelin pathway. *J. Clin. Invest.* **110**(1), 3-8 (2002).
8. Kornhuber, J., Medlin, A., Bleich, S., *et al.* High activity of acid sphingomyelinase in major depression. *J. Neural Transm.* **112**, 1583-1590 (2005).
9. Wascholowski, V., Giannis, A. Neutral sphingomyelinase as a target for drug design. *Drug News Perspect.* **14**(10), 581-590 (2001).
10. Clarke, C.J., Snook, C.F., Tani, M., *et al.* The extended family of neutral sphingomyelinases. *Biochemistry* **45**(38), 11247-11256 (2006).
11. Luberto, C., Hassler, D.F., Signorelli, P., *et al.* Inhibition of tumor necrosis factor-induced cell death in MCF7 by a novel inhibitor of neutral sphingomyelinase. *J. Biol. Chem.* **277**(43), 41128-41139 (2002).
12. Hakogi, T., Fujii, S., Morita, M., *et al.* Synthesis of sphingomyelin sulfur analogue and its behavior toward sphingomyelinase. *Bioorg. Medicinal Chem. Letters* **15**, 2141-2144 (2005).

Related Products

Cholesterol Assay Kit - Item No. 10007640
Cholesterol Cell-Based Detection Assay Kit - Item No. 10009779
FABP4 Inhibitor/Ligand Screening Assay Kit - Item No. 10010231
GW 4869 - Item No. 13127
β-Hydroxybutyrate (Ketone Body) Assay Kit - Item No. 700190
LDL Uptake Cell-Based Assay Kit - Item No. 10011125
oxLDL-β₂GPI (human) ELISA Kit - Item No. 10007893
Lipid Hydroperoxide (LPO) Assay Kit - Item No. 705002
Liver X Receptor β Transcription Factor Assay Kit - Item No. 10011119
PAF Acetylhydrolase Assay Kit - Item No. 760901
PAF Acetylhydrolase Inhibitor Screening Assay Kit - Item No. 10004380
Phosphatidylcholine Assay Kit - Item No. 10009926
PPARα Transcription Factor Assay Kit - Item No. 10006915
PPARα, δ, γ Complete Transcription Factor Assay Kit - Item No. 10008878
PPARδ Transcription Factor Assay Kit - Item No. 10006914
PPARγ FP-Based Ligand Screening Assay Kit - Green - Item No. 10007685
PPARγ Transcription Factor Assay Kit - Item No. 10006855
Sphingomyelin Assay Kit - Item No. 10009928
Sphingomyelinase Assay Kit - Item No. 10006964

Warranty and Limitation of Remedy

Cayman Chemical Company makes **no warranty or guarantee** of any kind, whether written or oral, expressed or implied, including without limitation, any warranty of fitness for a particular purpose, suitability and merchantability, which extends beyond the description of the chemicals hereof. Cayman **warrants only** to the original customer that the material will meet our specifications at the time of delivery. Cayman will carry out its delivery obligations with due care and skill. Thus, in no event will Cayman have **any obligation or liability**, whether in tort (including negligence) or in contract, for any direct, indirect, incidental or consequential damages, even if Cayman is informed about their possible existence. This limitation of liability does not apply in the case of intentional acts or negligence of Cayman, its directors or its employees.

Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.

12								
11								
10								
9								
8								
7								
6								
5								
4								
3								
2								
1								
	A	B	C	D	E	F	G	H

NOTES

This document is copyrighted. All rights are reserved. This document may not, in whole or part, be copied, photocopied, reproduced, translated, or reduced to any electronic medium or machine-readable form without prior consent, in writing, from Cayman Chemical Company.

©11/16/2011, Cayman Chemical Company, Ann Arbor, MI, All rights reserved. Printed in U.S.A.