Sphingosine Kinase 1 Inhibitor Screening Assay Kit

Item No. 700430

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, store kit at -20°C or individual components can be stored as stated below.

Item Number	Item	Quantity/Size	Storage
700431	SPHK Assay Buffer (10X)	1 vial/5 ml	-20°C
700432	SPHK Enzyme Mixture	2 vials	-20°C
700433	SPHK Fluorometric Detector	2 vials	-20°C
700434	SPHK Cofactor Mixture	2 vials	-20°C
700435	SPHK DMSO	1 vial/ 1 ml	Room temperature
700436	SPHK ATP	2 vials	-20°C
700437	SPHK Substrate	1 vial/600 µl	-20°C
700438	SPHK 1 (human recombinant)	1 vial/50 µl	-20°C
700439	SPHK ADP	1 vial	-20°C
400017	96-Well Plate (black)	1 plate	Room temperature
400012	96-Well Cover Sheet	1 cover	Room temperature

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

Email: 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 as directed in the **Materials Supplied** section on page 3 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 between 530-540 nm and an emission wavelength between 580-590 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

Sphingosine kinase (SPHK) is an important enzyme in the sphingolipid metabolic pathway. SPHKs phosphorylate D-*erythro*-sphingosine to yield sphingosine-1-phosphate (S1P). S1P exhibits a broad spectrum of biological activites including cell proliferation, survival, migration, cytoskeletal organization, and morphogenesis.[1-3] To date, two isoforms of SPHK, SPHK 1 and SPHK 2, have been identified and characterized from mammalian cells.[4] Among them, SPHK 1 has been implicated to play a role in signaling pathways in mast cells and in TNF-α-triggered responses of fibroblasts and epithelial cells. Recent findings also indicate that SPHK 1 mRNA levels are increased in a variety of human tumors. Brain tumors with high SPHK 1 expression correlated with poor survival of patients and high expression of SPHK 1 appears to be an oncogenic event required for erythroleukaemia progression.[5,6] Thus SPHK is a potential therapeutic target for the control of cancer and inflammation.[5,6] Recently, SPHK 1 has been shown to be up-regulated in stimulated human phagocytes and in peritoneal phagocytes of patients with severe sepsis.[7] It was also observed that treatment with a specific SPHK 1 inhibitor resulted in protection against sepsis in mice, suggesting that inhibition of SPHK 1 is a potential therapy for septic shock.[7]

About This Assay

Cayman's SPHK 1 Inhibitor Screening Assay Kit provides a convenient fluorescence-based method for screening SPHK 1 inhibitors. The procedure requires only two easy steps, both performed in the same microplate (Figure 1). In the first step, human recombinant SPHK 1, along with ATP, phosphorylates D-*erythro*-sphingosine C-18. Next, ADP is converted to hydrogen peroxide (H_2O_2) in a series of enzymatic reactions. H_2O_2 in the presence of ADHP and peroxidase yields the fluorescent product, resorufin. Resorufin can be analyzed with an excitation wavelength between 530-540 nm and an emission wavelength between 580-590 nm.

Step 1

D-*erythro*-sphingosine C-18 + ATP $\xrightarrow{\text{SPHK 1}}$ Sphingosine-1-phosphate + ADP

Step 2

ADP $\xrightarrow[\text{Cofactors}]{\text{Enzymes}}$ H_2O_2 $\xrightarrow[\text{Peroxidase}]{\text{ADHP}}$ Resorufin (Ex_{530}/Em_{590})

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. SPHK Assay Buffer (10X) - (Item No. 700431)

The vial contains 5 ml of buffer. Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water. This final Buffer (20 mM HEPES, pH 7.4, containing 50 mM sodium chloride, 10 mM magnesium chloride, 0.02% Triton X-100, and 1 mM EGTA) should be used in the assay and for diluting reagents. When stored at 4°C, this diluted buffer is stable for at least six months.

2. SPHK Enzyme Mixture - (Item No. 700432)

Each vial contains a lyophilized powder of various enzymes. Reconstitute the contents of the vial with 3 ml of diluted Assay Buffer and place on ice. The reconstituted enzyme mixture is stable for four hours on ice. One vial is enough enzyme mixture to assay 60 wells. Use the additional vial if assaying the entire plate.

3. SPHK Fluorometric Detector - (Item No. 700433)

The vials contain a clear lyophilized powder of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP). Immediately prior to assaying, add 200 µl of SPHK DMSO (Item No. 700435) to the vial and vortex. Add 800 µl of diluted Assay Buffer to the vial and vortex. The reconstituted mixture is stable for 60 minutes. After 60 minutes, increased background fluorescence will occur.

4. SPHK Cofactor Mixture - (Item No. 700434)

Each vial contains a lyophilized powder of various enzyme cofactors. Reconstitute the contents of the vial with 2.7 ml of diluted Assay Buffer and 300 µl of reconstituted Fluorometric Detector. One vial is enough Cofactor Mixture to assay 60 wells. Use the additional vial if assaying the entire plate. The reconstituted mixture is stable for 60 minutes. After 60 minutes, increased background fluorescence will occur.

5. SPHK DMSO - (Item No. 700435)

The vial contains 1 ml of dimethylsulfoxide (DMSO). The reagent is ready to use as supplied.

6. SPHK ATP - (Item No. 700436)

The vials contain a lyophilized powder of adenosine triphosphate (ATP). Reconstitute the contents of the vial with 2 ml of diluted Assay Buffer. One vial is enough ATP to assay 60 wells. Use the additional vial if assaying the entire plate. Reconstituted ATP is stable for four hours at room temperature.

7. SPHK Substrate - (Item No. 700437)

The vial contains 600 µl of 2 mM D-*erythro*-sphingosine C-18 in ethanol. Dilute 240 µl of substrate with 960 µl of diluted Assay Buffer and vortex. This is enough substrate to assay 60 wells. Prepare additional substrate as needed. The diluted Substrate is stable for four hours at room temperature. The addition of 20 µl to the assay yields a final concentration of 50 µM substrate. *NOTE: The K_m value for the substrate is 17.8 µM. This concentration may be reduced by dilution with diluted Assay Buffer at the user's discretion, particularly when assaying for competitive inhibitors.*

8. SPHK 1 (human recombinant) Assay Reagent - (Item No. 700438)

The vial contains 50 µl of human recombinant sphingosine kinase 1. Dilute 20 µl with 580 µl of diluted Assay Buffer and store on ice. The diluted enzyme is stable for four hours on ice. This diluted enzyme is enough to assay 60 wells. Prepare additional enzyme as needed.

9. SPHK ADP - (Item No. 700439)-*optional*

The vial contains a lyophilized powder of adenosine diphosphate (ADP). ADP can be used to screen for assay interference (see **Interference** section, on page 14).

ASSAY PROTOCOL

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 and three wells designated as Background Wells. We suggest that each inhibitor sample 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 compounds to be measured in triplicate is given below (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 160 µl in all the wells.
- All reagents except the Enzyme Mixture and SPHK 1 must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- We recommend assaying samples in triplicate, but it is the user's discretion to do so.
- The assay is performed at room temperature.
- 30 inhibitor samples can be assayed in triplicate or 45 in duplicate.
- Monitor the fluorescence with an excitation wavelength between 530-540 nm and emission wavelength between 580-590 nm.

Performing the Assay

1. **100% Initial Activity Wells** - add 50 µl of Cofactor Mixture, 50 µl of Enzyme Mixture, 10 µl of diluted SPHK 1, and 10 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
2. **Background Wells** - add 50 µl of Cofactor Mixture, 50 µl of Enzyme Mixture, 10 µl of diluted Assay Buffer, and 10 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
3. **Inhibitor Wells** - add 50 µl of Cofactor Mixture, 50 µl of Enzyme Mixture, 10 µl of diluted SPHK 1, and 10 µl of Inhibitor* to three wells.
4. Initiate the reactions by quickly adding 20 µl of diluted Substrate solution and then 20 µl of ATP to all the wells being used.
5. Cover the plate with the plate cover and incubate for 10 minutes at room temperature.
6. Remove the plate cover and read the fluorescence using an excitation wavelength between 530-540 nm and an emission wavelength between 580-590 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples.

*Inhibitors can be dissolved in diluted Assay Buffer, ethanol, or dimethylsulfoxide and should be added to the assay in a final volume of 10 µl. Methanol reduced enzyme activity by 13% and thus it is not recommended for dissolving inhibitors. In the event that the appropriate concentration of inhibitor needed for SPHK 1 inhibition is completely unknown, we recommend that several concentrations of the compound be assayed. For determination of IC_{50} values, use multiple concentrations of inhibitor to cover a large range.

Well	Cofactor Mixture (µl)	Enzyme Mixture (µl)	SPHK 1 (µl)	Solvent (µl)	Inhibitor (µl)	Assay Buffer (µl)
100% Initial Activity	50	50	10	10	-	-
Background	50	50	-	10	-	10
Inhibitor	50	50	10	-	10	-

Table 1. Pipetting summary

ANALYSIS

Calculations

1. Determine the average fluorescence of 100% Initial Activity, Background, and Inhibitor wells.
2. Subtract the fluorescence of the background wells from the fluorescence of the 100% Initial Activity and Inhibitor wells.
3. Determine the percent inhibition or percent Initial Activity for each compound.

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

$$\% \text{ Initial Activity} = \left[\frac{\text{Inhibitor}}{\text{Initial Activity}}\right] \times 100$$

4. If multiple concentrations of inhibitor are tested, graph either 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). An example of SPHK 1 inhibition by SPHK Inhibitor 2 (Item No. 10009222) is shown in Figure 3, on page 13.[8]

Performance Characteristics

Precision:

When a series of 16 SPHK 1 measurements were performed on the same day, the intra-assay coefficient of variation was 3.2%. When a series of 16 SPHK 1 measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 3.3%.

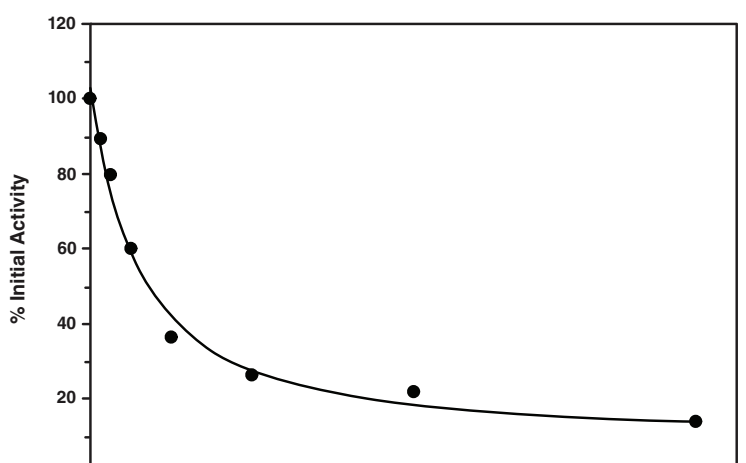


Figure 3. Inhibition of SPHK 1 by SPHK Inhibitor 2 (IC_{50} = 1.5 µM)

Interferences

A. It is possible that a compound/inhibitor can exhibit fluorescence or cause a decrease in the fluorescence. To correct for this effect, assay each inhibitor concentration in a background well (*i.e.*, substitute inhibitor for solvent in background wells) and then subtract the fluorescence value from the corresponding inhibitor well to yield the corrected inhibitor value. Use this value in the calculation to determine the percent inhibition or percent initial activity of the inhibitor (See **Calculations** section).

B. It is possible that a compound tested for SPHK 1 inhibition is actually interfering with the development of the assay and is not really an inhibitor of SPHK 1. Potential coupling enzyme interference can be tested by assaying the compound in question with ADP. A procedure is outlined below.

Testing for Coupling Enzyme Interference

1. Reconstitute SPHK ADP (Item No. 700439) with 1 ml of diluted Assay Buffer. Reconstituted ADP is stable for four hours at room temperature.
2. **ADP wells** - add 50 µl of Cofactor Mixture containing ADHP, 10 µl of ADP, and 10 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
3. **Compound wells** - add 50 µl of Cofactor Mixture containing ADHP, 10 µl of ADP, and 10 µl of compound to three wells.
4. Initiate the reactions by adding 50 µl of Enzyme Mixture to all the wells being used.
5. Cover the plate with the plate cover and incubate for 10 minutes at room temperature.
6. Remove the plate cover and read the fluorescence using an excitation wavelength between 530-540 nm and an emission wavelength between 580-590 nm. It may be necessary to adjust the gain setting on the instrument to allow for the measurement of all the samples.

Calculating the percent interference

1. Determine the average fluorescence of each ADP and compound wells.
2. Determine the percent interference for the compound. To do this, subtract each compound value from the ADP value. Divide the result by the ADP value and then multiply by 100 to give the percent interference. The percent interference should be less than 10% for the compound to be not affecting the assay.

$$\% \text{ Interference} = \left[\frac{\text{ADP value - Compound value}}{\text{ADP value}}\right] \times 100$$

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 above background is seen in the Inhibitor wells	Inhibitor concentration is too high and inhibited all of the enzyme activity	Reduce the concentration of the inhibitor and re-assay
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read
No inhibition was seen with the inhibitor	A. The inhibitor concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay

References

1. Takuwa, Y., Takuwa, N., and Sugimoto, N. The Edg family G protein-coupled receptors for lysophospholipids: Their signaling properties and biological activities. *J. Biochem.* **131**, 767-771 (2002).
2. Ishii, I., Fukushima, N., Ye, X., *et al.* Lysophospholipid receptors: Signaling and biology. *Annu. Rev. Biochem.* **73**, 321-354 (2004).
3. Kluk, M.J. and Hla, T. Signaling of sphingosine-1-phosphate *via* the S1P/EDG-family of G-protein-coupled receptors. *Biochim. Biophys. Acta* **1582**, 72-80 (2002).
4. Pitson, S.M., D'Andrea, R.J., Vandeleur, L., *et al.* Human sphingosine kinase: Purification, molecular cloning and characterization of the native and recombinant enzymes. *Biochem. J.* **350**, 429-441 (2000).
5. Taha, T.A., Hannun, Y.A., and Obeid, L.M. Sphingosine kinase: Biochemical and cellular regulation and role in disease. *J. Biochem. Mol. Biol.* **39(2)**, 113-131 (2006).
6. Leclercq, T.M. and Pitson, S.M. Cellular signalling by sphingosine kinase and sphingosine 1-phosphate. *IUBMB Life* **58**, 467-472 (2006).
7. Puneet, P., Yap, C.T., Wong, L., *et al.* SphK1 regulates proinflammatory responses associated with endotoxin and polymicrobial sepsis. *Science* **328**, 1290-1294 (2010).
8. French, K.J., Schrecengost, R.S., Lee, B.D., *et al.* Discovery and evaluation of inhibitors of human sphingosine kinase. *Cancer Res.* **63**, 5962-5969 (2003).

Related Products

CAY10621 - Item No. 13371
FTY720 - Item No. 10006292
D-*erythro*-Sphingosine C-18 - Item No. 10007907
Sphingosine Kinase 1 (human recombinant) - Item No. 10009236
Sphingosine Kinase 2 (human recombinant) - Item No. 10009237
Sphingosine Kinase Inhibitor 2 - Item No. 10009222
Sphingosine-1-phosphate - Item No. 62570

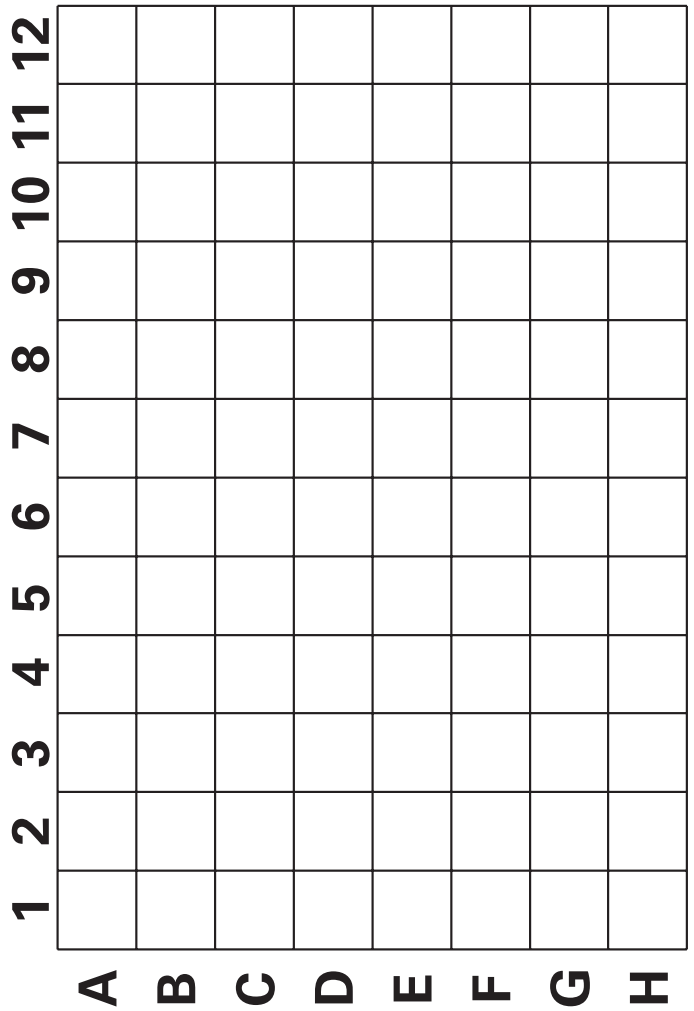
Warranty and Limitation of Remedy

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.



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