Sphingosine Kinase 2 Inhibitor Screening Assay Kit

Item No. 701870

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TABLE OF CONTENTS

GENERAL INFORMATION
3 Materials Supplied
4 Safety Data
4 Precautions
4 If You Have Problems
5 Storage and Stability
5 Materials Needed but Not Supplied

INTRODUCTION
6 Background
7 About This Assay

PRE-ASSAY PREPARATION
8 Sample Preparation
8 Reagent Preparation

ASSAY PROTOCOL
10 Plate Set Up
12 Performing the Assay

ANALYSIS
14 Calculations
15 Performance Characteristics

RESOURCES
19 Troubleshooting
20 References
21 Plate Template
22 Notes
23 Warranty and Limitation of Remedy

GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening the kit, store individual components as stated below.

Item Number	Item	Quantity/Size	Storage
701873	SPHK2 Assay Buffer (1X)	1 vial/14 ml	-20°C
701871	SPHK2 Enzyme (human, recombinant)	1 vial/50 µl	-80°C
701743	SPHK Substrate	2 vials	-80°C
700001	DMSO Assay Reagent	1 vial/1 ml	RT
701874	SPHK2 ATP	1 vial/5 ml	-20°C
701872	SPHK1/2 Inhibitor (SLC5111312)	1 vial	-20°C
400115	Half-Area 96-Well Solid Plate (black, clear bottom)	1 plate	RT
400023	Foil Plate Cover	1 ea	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

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

Email: techserv@caymanchem.com

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 (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure fluorescence with excitation and emission wavelengths of 550 and 584 nm, respectively. *NOTE: If a fluorescent plate reader is not available, the absorbance at 550 nm can be measured instead. However, in this case the signal-to-background ratio will be significantly lower compared to the fluorometric method.*
2. Adjustable pipettes; multichannel or repeating pipettor recommended

INTRODUCTION

Background

Sphingosine kinase 1 (SPHK1) and SPHK2 phosphorylate D-*erythro*-sphingosine to yield the bioactive lipid mediator sphingosine-1-phosphate (S1P). The preferred substrate for SPHK2 is D-*erythro*-dihydrosphingosine, and it also phosphorylates D,L-*threo*-dihydrosphingosine.[1-3] SPHK2 is located in the nucleus, endoplasmic reticulum, and mitochondria.[1,4,5] S1P formed by SPHK2 in the nucleus induces gene transcription by inhibiting histone deacetylase 1 (HDAC1) and HDAC2, whereas S1P synthesized by SPHK2 in the ER is converted to ceramide, and S1P made in the mitochondria induces apoptosis by interacting with Bcl/xL. Unlike SPHK1, SPHK2-derived S1P increases apoptosis and inhibits cell growth *in vitro*.[4-6] *In vivo*, hepatic SPHK2 regenerates S1P intracellularly after its dephosphorylation to sphingosine by the phospholipid phosphatase PLPP3, which removes a portion of the sphingosine from the circulation. Therefore, inhibition of SPHK2 results in a paradoxical increase in circulating S1P levels as sphingosine returns to the circulation where it can be phosphorylated by SPHK1.[7] SPHK2 levels in tumors from patients with non-small cell lung cancer (NSCLC) are positively correlated with cancer progression and associated with lower disease-free and overall survival.[8] The development of SPHK2-specific inhibitors is important to clarify the role of SPHK2 in cancer and other diseases.

About This Assay

Cayman's SPHK2 Inhibitor Screening Assay Kit provides a robust and easy-to-use platform for identifying novel inhibitors of human SPHK2, an enzyme with roles in cell proliferation, apoptosis, and epigenetic regulation of gene expression. The assay uses an SPHK-specific fluorogenic substrate, NBD-sphingosine. SPHK2 phosphorylates this substrate generating a shift in its spectral properties, which can be easily quantified using a fluorescence plate reader at excitation and emission wavelengths of 550 and 584 nm, respectively. The reversible dual SPHK1/2 inhibitor SLC5111312 is included as a positive control and inhibits SPHK2 with an IC_{50} value of approximately 4 µM.

PRE-ASSAY PREPARATION

Sample Preparation

All inhibitors, be they small molecules, natural products, or proteins, should be prepared in SPHK2 Assay Buffer (1X) at a concentration 20X the desired final assay concentration (*e.g.*, for 1 µM final assay concentration, a 20 µM stock should be made). This solution may contain up to 100% DMSO or short-chain alcohols (*e.g.*, MeOH, EtOH). The final concentration of organic solvents in the assay will then be ≤5% (see 'Effects of Solvents' on page 18). The use of dimethyl formamide (DMF) is not recommended.

Reagent Preparation

NOTE: Do not add SPHK Substrate or SPHK2 Enzyme (human, recombinant) to SPHK2 Assay Buffer (1X) until just prior to use.

NOTE: The volumes indicated below are sufficient for assaying 48 wells.

1. **SPHK2 Assay Buffer (1X)**

   SPHK2 Assay Buffer (1X) (Item No. 701873) is ready to use as supplied. Once thawed, the SPHK2 Assay Buffer (1X) may be stored at 4°C for at least 1 month.

2. **SPHK Substrate**

   Thaw and then briefly centrifuge one vial of SPHK Substrate (Item No. 701743). The substrate should appear golden yellow in color. Add 6 ml of SPHK2 Assay Buffer (1X) to a clean tube, saving the remaining SPHK2 Assay Buffer (1X) in the original tube for use in the background (no ATP) wells (see 'Performing the Assay', step 3, on page 12). Add 165 µl of SPHK Substrate to the 6 ml of SPHK2 Assay Buffer (1X) within 5 minutes of thawing.

3. **SPHK2 Enzyme (human, recombinant)**

   Thaw and then briefly centrifuge SPHK2 Enzyme (Item No. 701871) to ensure that all contents are at the bottom. Add 25 µl of SPHK2 Enzyme (human, recombinant) to the SPHK2 Assay Buffer (1X) containing SPHK Substrate (step 2, above) immediately prior to performing the assay. This is the Complete SPHK2 Assay Buffer (see 'Performing the Assay', step 2, on page 12), which is stable on ice for two hours. The undiluted enzyme can be stored at -80°C, limiting freeze-thaw cycles.

4. **SPHK1/2 Inhibitor (SLC5111312)**

   This vial contains 100 µl of 2 mM SPHK1/2 Inhibitor (SLC5111312) (Item No. 701872) in DMSO, which can be used as a positive control. The SPHK1/2 Inhibitor (SLC5111312) is ready to use as supplied. Thaw the SPHK1/2 Inhibitor (SLC5111312) and keep it at room temperature until used. If all of the SPHK1/2 Inhibitor (SLC5111312) will not be used at one time, aliquot the undiluted inhibitor and store at -20°C.

5. **ATP**

   Thaw the vial of SPHK2 ATP (Item No. 701874) and then keep on ice until used.

6. **DMSO Assay Reagent**

   The DMSO Assay Reagent (Item No. 700001) is ready to use as supplied.

ASSAY PROTOCOL

Plate Set Up

The 96-well plate included with this kit is supplied ready to use. 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. It is suggested that each inhibitor, including the positive control SPHK1/2 Inhibitor (SLC5111312), be assayed in triplicate. It is suggested that the contents of each well be recorded on the template sheet provided on page 21. A typical layout of samples to be measured in triplicate is shown in Figure 1.

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

BW - Background Wells
A - 100% Initial Activity Wells
PC - Positive Control Wells
1-29 - Sample Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette 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.
- SPHK2 Assay Buffer (1X) contains detergent. Avoid vigorous mixing that will produce excessive bubbles. Any bubbles large enough to interfere with signal detection can be broken using a needle.

General Information

- The final volume of the assay is 100 µl in all the wells.
- Use SPHK2 Assay Buffer (1X) in the assay.
- All reagents should be prepared as described above and the assay buffer should be kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time. The two vials of substrate are provided for use in two separate 48-well assays or one 96-well assay.
- It is recommended to assay the samples in triplicate, but it is at the user's discretion to do so.
- The assay is performed at room temperature.
- Monitor the fluorescence with an excitation wavelength of 550 nm and an emission wavelength of 584 nm or, alternatively, monitor absorbance at 550 nm.
- If fluorescence is monitored kinetically, it is highly recommended that several test wells with no inhibitor be run prior to setting up a large assay so that the fluorometer gain setting can be adjusted appropriately so as not to max out the signal.

Performing the Assay

1. Prior to adding SPHK Substrate and SPHK2 Enzyme (human, recombinant) to SPHK2 Assay Buffer (1X), ensure all test compounds are diluted and ready for addition to the reactions.
2. Add 75 µl of the Complete SPHK2 Assay Buffer to all wells being used (see 'Reagent Preparation', step 3, on page 9). The final substrate concentration in the reaction is 25 µM.
3. **Background Wells**: add 5 µl DMSO Assay Reagent and 20 µl SPHK2 Assay Buffer (1X) to three wells. Mix thoroughly without introducing bubbles. If a solvent other than DMSO is used to prepare the unknown inhibitors, add 5 µl of that solvent and 20 µl SPHK2 Assay Buffer (1X) to three additional wells as background wells for that solvent.
4. **100% Initial Activity Wells**: add 5 µl of DMSO Assay Reagent to three wells. If a solvent other than DMSO is used to prepare the unknown inhibitors, add 5 µl of that solvent to three additional wells as 100% initial activity wells for that solvent.
5. **Inhibitor/Positive Control Wells**: add 5 µl of SPHK1/2 Inhibitor (SLC5111312) to three wells. Add 5 µl unknown inhibitor to the inhibitor wells.
6. Add 20 µl ATP to all wells, except background wells, and mix thoroughly without introducing bubbles.
7. Cover the plate with the Foil Plate Cover (Item No. 400023) and incubate for 90 minutes at room temperature.
8. Remove the plate cover and read the plate with an excitation wavelength of 550 nm and an emission wavelength of 584 nm. It may be necessary to adjust the gain setting to allow for the measurement of all samples.* Alternatively, the absorbance may be read at 550 nm but the signal-to-background ratio will be significantly lower compared to the fluorometric method.

*If desired, the assay may be read kinetically rather than as an endpoint. Reading the assay kinetically may increase the signal-to-background ratio. The fluorescence should be measured at least once every two minutes at room temperature for 90 minutes. Determine the initial rate based on the linear portion of the kinetic curve. Calculations can be performed as shown below substituting initial rates for average fluorescence.

ANALYSIS

Calculations

1. Determine the average fluorescence (AF) of each sample.
2. Subtract the AF of the background wells from the AF of the 100% initial activity and inhibitor wells. These are the corrected values.
3. Determine the percent inhibition or percent activity for each inhibitor using one of the following equations:

$$\%\text{Inhibition} = \left[\frac{(\text{corrected 100\% initial activity} - \text{calculated inhibitor activity})}{\text{corrected 100\% initial activity}}\right] \times 100$$

$$\%\text{Activity} = \left[\frac{(\text{corrected inhibitor activity})}{\text{corrected 100\% initial activity}}\right] \times 100$$

4. Graph the percent inhibition or percent activity as a function of inhibitor concentration to determine the IC_{50} value (the concentration at which there is 50% inhibition) of the inhibitor. Inhibition of recombinant human SPHK2 by SPHK1/2 Inhibitor (SLC5111312) is shown in Figure 2 (see page 16).

Performance Characteristics

Z' Factor:

Z' factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[9]

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{|\mu_{c+} - \mu_{c-}|}$$

Where σ: Standard deviation
μ: Mean
c+: Positive control
c-: Negative control

The theoretical upper limit for the Z' factor is 1.0. A robust assay has a Z' factor >0.5. The Z' factor for Cayman's SPHK2 Inhibitor Screening Assay Kit was determined to be 0.97.

Sample Data:

The data presented are an example of data typically produced with this kit; however, your results will not be identical to these. Do not use the data below to directly compare to your samples. Your results could differ substantially.

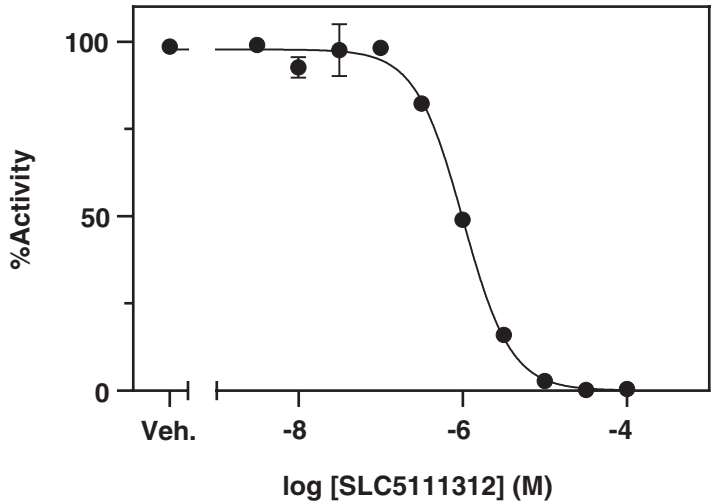

Figure 2. Inhibition of recombinant human SPHK2 by SPHK1/2 Inhibitor (SLC5111312). Data are plotted as the mean of duplicate measurements ± the standard deviation. The vehicle/solvent control (Veh.) represents 100% initial activity. The IC_{50} value of SPHK1/2 Inhibitor (SLC5111312) in this example is 4 µM.

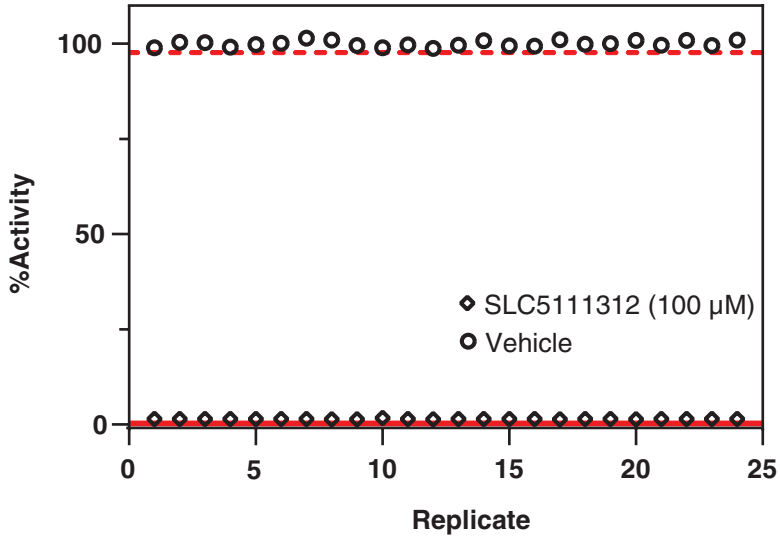

Figure 3. Typical Z′ data for the SPHK2 Inhibitor Screening Assay Kit. Data are shown from 24 replicates each for vehicle control (Veh.) and 100 µM SPHK1/2 Inhibitor (SLC5111312) prepared as described in the kit booklet. The calculated Z′ factor for this experiment was 0.97. The red lines correspond to three standard deviations from the mean of each replicate set.

Effects of Solvents:

Compounds may be prepared in organic solvents such as DMSO or short-chain alcohols (*e.g.*, MeOH, EtOH), as long as the final concentration of organic solvents in the assay is ≤5%. DMF is not a recommended solvent for this assay. A titration of DMF showed that the signal increases with increasing solvent concentration. The proper vehicle control should always be included in the assay.

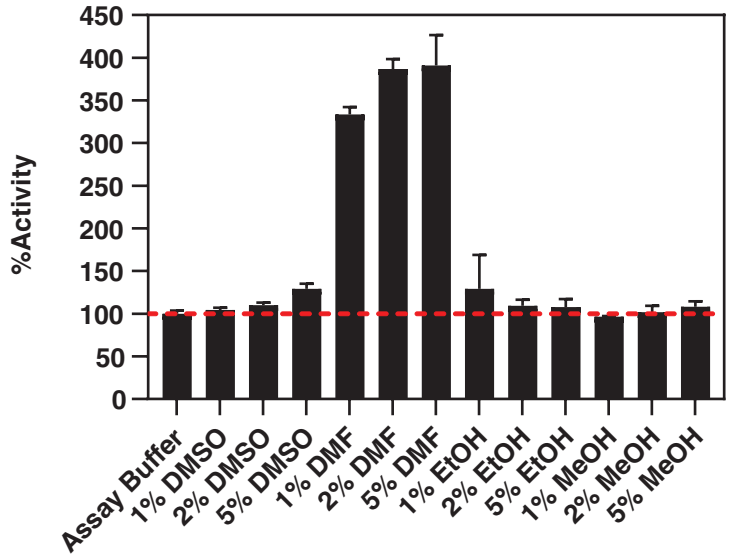

Figure 4. The effect of solvent on the readout of SPHK2 activity. The data are shown as the mean ± standard deviation for duplicate reactions containing the indicated concentration of solvents. The red dotted line corresponds to the mean of the reactions containing assay buffer in place of any solvent.

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. Break any large bubbles with a needle
No fluorescence detected above background in the inhibitor wells	A. Either substrate or enzyme was not added to the wells B. Inhibitor concentration is too high and inhibited all of the enzyme activity	A. Make sure to add all the components to the well(s) and re-assay B. Reduce the inhibitor concentration 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 seen with compound	A. The compound concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the compound concentration and re-assay

References

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3. Liu, H., Sugiura, M., Nava, V.E., *et al.* Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. *J. Biol. Chem.* **275(26)**, 19513-19520 (2000).
4. Liu, H., Toman, R.E., Goparaju, S.K., *et al.* Sphingosine kinase type 2 is a putative BH3-only protein that induces apoptosis. *J. Biol. Chem.* **278(41)**, 40330-40336 (2003).
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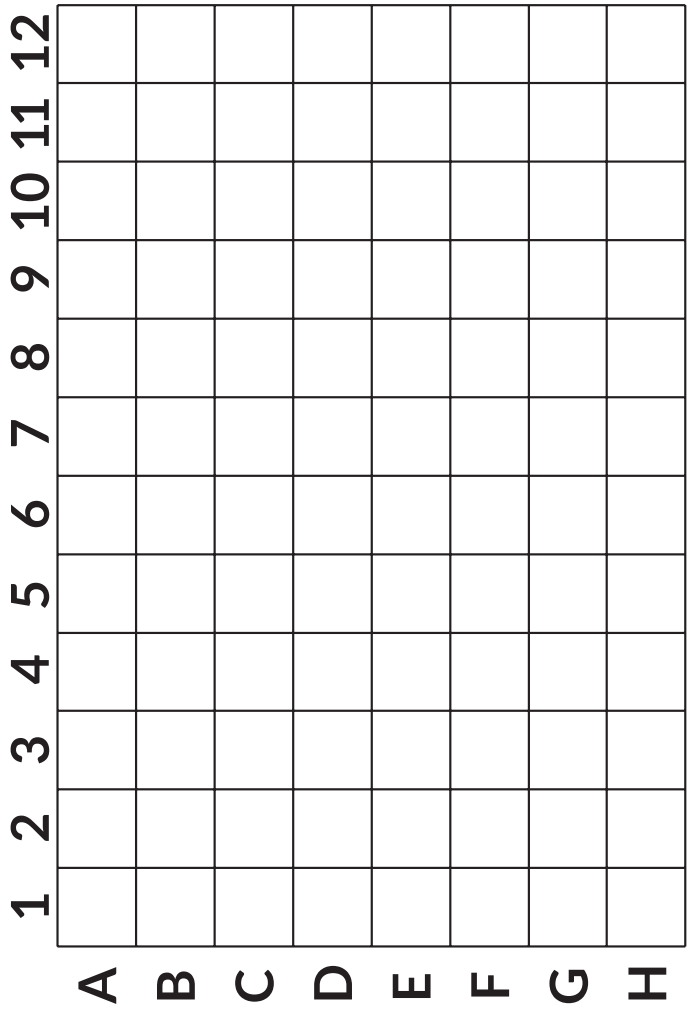

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.