Sphingosine Kinase 1 Inhibitor Screening Assay Kit

Item No. 701740

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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
701741	SPHK1 Assay Buffer (1X)	1 vial/14 ml	-20°C
701742	SPHK1 Enzyme (human, recombinant)	1 vial/50 µl	-80°C
701743	SPHK Substrate	2 vials/300 µl	-80°C
700001	DMSO Assay Reagent	1 vial/1 ml	RT
701744	SPHK1 ATP	1 vial/5 ml	-20°C
701745	SPHK1 Inhibitor (PF-543)	1 vial/100 µl	-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 sphingosine-1-phosphate (S1P).[1,2] SPHK1 is located in the cytosol and activated by phosphorylation at serine 225.[1,3] Upon activation, it translocates to the plasma membrane where it catalyzes the phosphorylation of sphingosine to form S1P.[1] S1P acts intracellularly or is secreted outside of the cell where it activates the S1P receptors, $S1P_1$-$S1P_5$, in an autocrine or paracrine manner initiating processes such as cell proliferation, angiogenesis, and cell migration.[1,4] Overexpression of *SPHK1* is associated with metabolic reprogramming of cancer cells *in vitro* and increases tumor growth *in vivo* in mouse models, whereas genetic knockout reduces the incidence of tumors in mouse models.[5,6] SPHK1 is overexpressed in a variety of cancer cells, and the expression level is associated with poor prognosis, lower survival rates, and metastasis.[1,7-9] It is also an independent predictor of distant metastasis in patients with colorectal or triple-negative breast cancer.[7,10] Inhibitors of SPHK1 have been developed that display efficacy *in vitro* and in animal models of cancer through multiple mechanisms. In contrast, activation of SPHK1 decreases sphingoid base, sphingolipid, and cholesterol accumulation in an *in vitro* model of Niemann-Pick type C (NPC) disease.[11] The development of additional SPHK1 inhibitors for use in cancer is warranted, while SPHK1 activators may have utility in the treatment of sphingolipid and cholesterol metabolism disorders.

About This Assay

Cayman's SPHK1 Inhibitor Screening Assay Kit provides a robust and easy-to-use platform for identifying novel inhibitors of human SPHK1, an enzyme implicated in the control of diverse biological functions including cell proliferation, survival and differentiation, cell migration and tissue invasion, and angiogenesis and vascular integrity. The assay uses an SPHK-specific fluorogenic substrate, NBD-sphingosine. SPHK1 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 potent and reversible SPHK1 inhibitor PF-543 is included as a positive control and inhibits SPHK1 with an IC_{50} value of 60.5 ± 34.3 nM.

PRE-ASSAY PREPARATION

Sample Preparation

All inhibitors, be they small molecules, natural products, or proteins, should be prepared in SPHK1 Assay Buffer (1X) at a concentration 20X the desired final assay concentration (*e.g.*, for 100 nM final assay concentration, a 2,000 nM stock should be made). This solution may contain up to 5% DMSO or dimethyl formamide (DMF). The use of short-chain alcohols (*e.g.*, MeOH, EtOH) is not recommended (see 'Effects of Solvents' on page 19).

Reagent Preparation

NOTE: Do not add SPHK Substrate or SPHK1 Enzyme (human, recombinant) to SPHK1 Assay Buffer (1X) until just prior to use. See 'Performing the Assay', step 2, on page 12.

1. **SPHK1 Assay Buffer (1X)**

   SPHK1 Assay Buffer (1X) (Item No. 701741) is ready to use as supplied. Once thawed, the SPHK1 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. It is recommended that the substrate be added to the master mix immediately prior to performing the assay. If all of the SPHK Substrate will not be used at one time, aliquot the undiluted substrate and store at -80°C where it will be stable for at least 6 months.

3. **SPHK1 Enzyme (human, recombinant)**

   SPHK1 Enzyme (Item No. 701742) should be thawed on ice and mixed prior to use. It is recommended that the enzyme be added to the master mix immediately prior to performing the assay. The diluted enzyme loses 20% of its activity when stored on ice for 4 hours. The undiluted enzyme can be aliquoted and stored at -80°C, limiting freeze-thaw cycles.

4. **SPHK1 Inhibitor (PF-543)**

   This vial contains 100 µl of 200 µM SPHK1 Inhibitor (PF-543) (Item No. 701745) in DMSO, which can be used as a positive control. The SPHK1 Inhibitor (PF-543) is ready to use as supplied. Thaw the SPHK1 Inhibitor (PF-543) and keep it at room temperature until used. If all of the SPHK1 Inhibitor (PF-453) will not be used at one time, aliquot the undiluted inhibitor and store at -20°C.

5. **ATP**

   Thaw the vial of SPHK1 ATP (Item No. 701744) 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(s) 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 Inhibitor (PF-543), be assayed in triplicate. It is suggested that the contents of each well be recorded on the template sheet provided on page 22. 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.
- SPHK1 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 SPHK1 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. Ensure all test compounds are diluted and ready for addition to the reactions.
2. Prepare a master mix containing SPHK1 Enzyme (human, recombinant) and SPHK Substrate in SPHK1 Assay Buffer (1X) according to Table 1, on page 13. Scale up or down as needed.

	Master Mix	
Reagent	50 wells	100 wells
SPHK Assay Buffer (1X)	4,325 µl	8,650 µl
SPHK Substrate	150 µl	300 µl
SPHK Enzyme	25 µl	50 µl
Total Volume:	4,500 µl	9,000 µl

Table 1. Preparation of the master mix

3. **Master Mix**: add 75 µl of the master mix to all wells.
4. **Background Wells**: add 5 µl DMSO Assay Reagent and 20 µl SPHK1 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 SPHK1 Assay Buffer (1X) to three additional wells as background wells for that solvent.
5. **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.
6. **Inhibitor/Positive Control Wells**: add 5 µl of SPHK1 Inhibitor (PF-543) to three wells. Add 5 µl unknown inhibitor to the inhibitor wells.

7. Add 20 µl ATP to all wells, except background wells, and mix thoroughly without introducing bubbles.
8. Cover the plate with the Foil Plate Cover (Item No. 400023) and incubate for 60 minutes at room temperature.
9. 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 60 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 SPHK1 by SPHK1 Inhibitor (PF-543) is shown in Figure 2 (see page 17).

Performance Characteristics

Z' Factor:

Z' factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[12]

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

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 SPHK1 Inhibitor Screening Assay Kit was determined to be 0.83.

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.

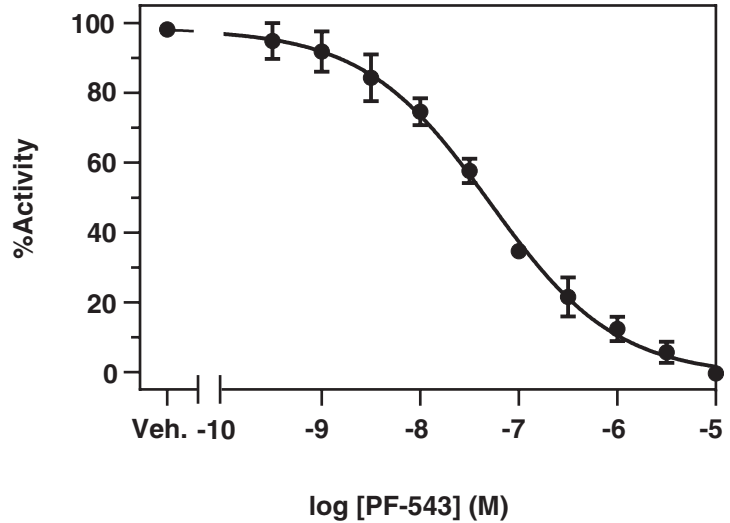

Figure 2. Inhibition of recombinant human SPHK1 by SPHK1 Inhibitor (PF-543). Data are plotted as the mean of duplicate measurements ± the standard deviation. The vehicle/solvent control (Veh.) represents 100% initial activity. The IC_{50} value of SPHK1 Inhibitor (PF-543) in this example is 51 nM.

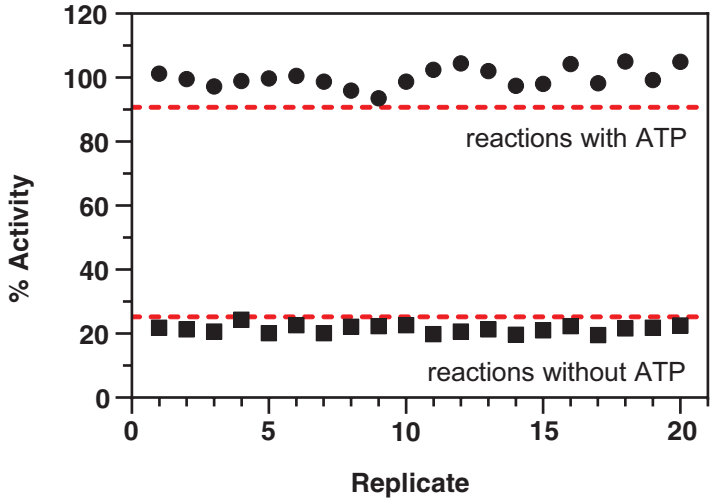

Figure 3. Typical Z′ data for the SPHK1 Inhibitor Screening Assay Kit. Data are shown from 20 replicates each for 100% initial activity (+ATP) wells and background activity (-ATP) for background wells prepared as described in the kit booklet. The calculated Z' factor for this experiment was 0.83. The red dotted 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 DMF as long as the final concentration of organic solvents in the assay is ≤5%. Short-chain alcohols (*e.g.*, MeOH, EtOH) are not recommended solvents for this assay. A titration of short-chain alcohols showed that the signal decreases with increasing solvent concentration. The proper vehicle control should always be included in the assay.

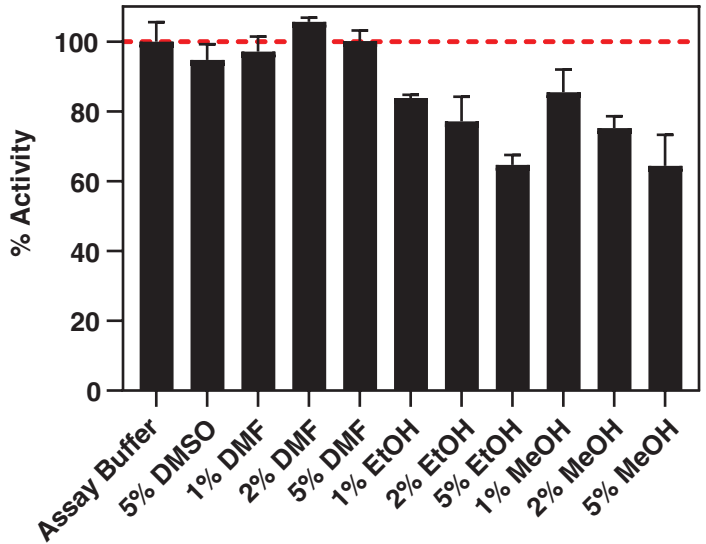

Figure 4. The effect of solvent on the readout of SPHK1 activity. The data are shown as the mean ± standard deviation for duplicate reactions containing the indicated concentration of solvents. The red dotted line corresponds to the mean of the reactions containing assay buffer in place of any solvent.

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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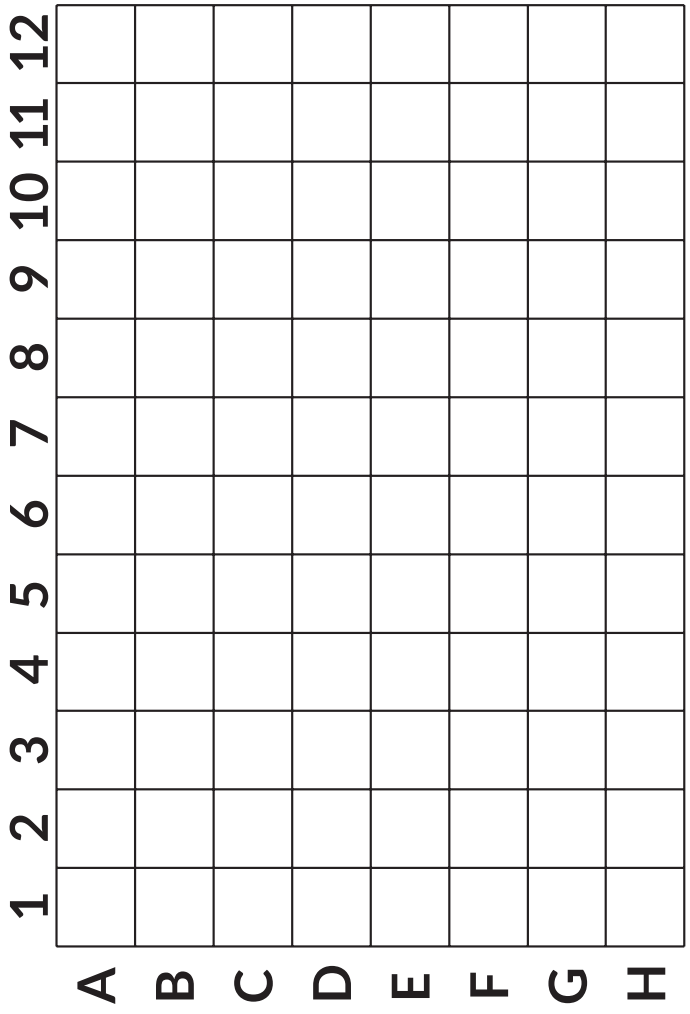

NOTES

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.