Echelon Biosciences Inc.

Sphingosine Kinase Activity Assay

K-3500 (96 tests)

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Description: Luminescent assay that measures Sphingosine Kinase activity in cell lysates and tissue homogenate samples through the measuremnet of ATP. This kit has been used to determine inhibition of SPHK1 and SPHK2 with recombinant enzymes.

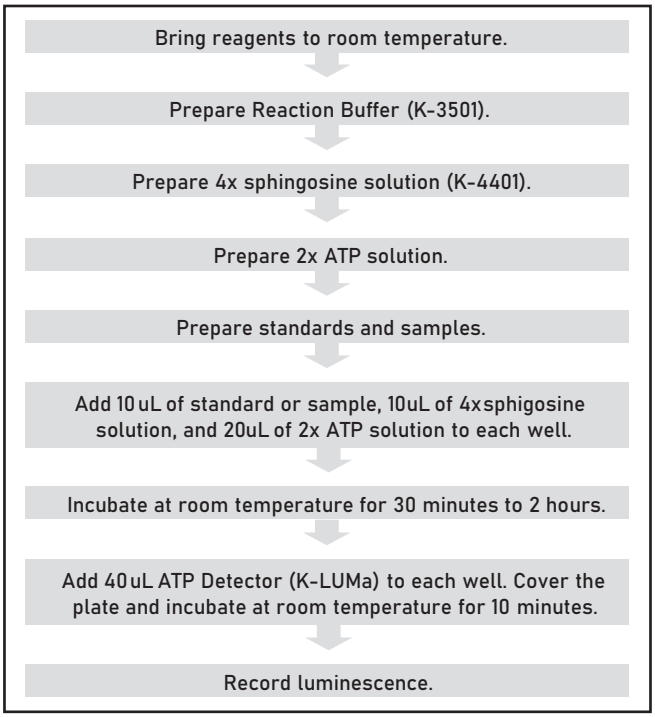
Materials Provided

Catalog #	Description	Quantity
K-3501	Reaction Buffer (12 mL)	1 bottle
K-4401	10 mM Sphingosine (100µL)	1 vial
K-LUMa	ATP Detector (5 mL)	1 bottle
K-ATP1	10 mM ATP (50µL)	1 vial
K-DTT1	50 µmol dried dithiothreitol (DTT) powder	1 vial
---	96-well round bottom white plate	1 each
---	Microtiter Plate Seal	1 each

Additional Materials Provided by User

- Source of purified sphingosine kinase (Cat. # E-K069, E-K068) as positive control (optional)
- Plate reader capable of reading luminescence in 96-well microtiter plates
- Microcentrifuge tubes (0.5 mL or 1.5 mL) or reservoir for standard dilution or sample loading
- Multichannel pipettes or automatic pipetting station

Quick Protocol



Storage

Upon receipt, the kit should be stored at -20°C. Under proper storage conditions, the kit components should remain stable for at least 6 months from date of receipt. Allow the reagents to warm to room temperature before opening vials.

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Background

Sphingosine kinases (SPHKs) catalyze the phosphorylation of sphingosine to sphingosine-1-phosphate (S1P). SPHKs have been implicated in proliferation, survival, migration and regulation of Ca2+ homoeostasis, development and regulation of the cardiovascular and nervous systems, inflammation, immunity, and cancer growth. There are two currently known SPHK isoforms, SPHK1 and SPHK2. It is believed that SPHK1 and SPHK2 have opposite functions, promoting cell growth and apoptosis respectively.

Assay Design

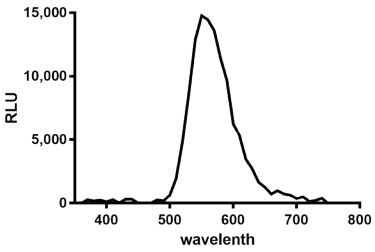
Echelon's Sphingosine Kinase Activity Assay is an ATP depletion assay which quantifies the remaining ATP levels in solution following the kinase reaction. The assay reaction is prepared by the addition of sphingosine kinase, sphingosine (substrate) in reaction buffer and initiated by the addition of ATP. The reaction is then stopped by adding the ATP detector after the chosen reaction time. The luminescent signal is inversely correlated with the kinase activity. Echelon's sphingosine kinase activity assay is tested on Echelon's SPHK1 (E-K068) and SPHK2 (E-K069).

Assay Notes

1. The ATP detector's (K-LUMa) linear range of detection may vary between instruments. Depending on the instrument capacity and sensitivity setting, the optimal range and sensitivity for the ATP detector (K-LUMa) is up to 100 µM.

2. The luminescent signal, or light, collected by your plate reader is not restricted to a particular wavelength. When setting up your instrument, select the option on your reader that allows the instrument to collect all wavelengths. For most plate readers this setting is labeled "all wavelengths". For BMG Labtech plate readers the "lens" setting will read all wavelengths. Please refer to your plate reader's manual and/or the manufacturer of your reader if you have questions on how to set up your specific instrument. If your instrument does not have this setting, or is limited to a certain filter, choose a broad filter centered at 550nm (Figure 1). When the luminescent signal is read at one wavelength, a lower signal may be observed.

Figure 2 I Wavelength Emission Spectra of ATP detector; K-LUMa



3. The buffer of your sample may interfere with the luminescent output. Sample buffers should always be included in the ATP standard curve and as a "buffer only" background control.

4. The assay has been tested with two commercially available lysis buffers (Figure 2) and common lysis buffer components (Figure 3). Since some buffers and lysis buffer components affect the assay, we suggest using Buffer 2 or the cells can be lysed with sonication and freeze thaw cycles in the provided reaction buffer (K-3501). If you plan to use your own lysis buffer we suggest testing it in the assay for interference before running samples. If your lysis buffer has a strong effect on the assay, change lysis buffer or dilute sample with reaction buffer (K-3501).

5. Each sample reaction contains 10 µL of sample (SPHK), 10 µL of sphingosine, and 20 µL ATP for a 40 µL reaction volume. The ATP standards contain 10 µL reaction buffer or sample buffer, plus 10 µL sphingosine and 20 µL ATP standard for a 40 µL reaction volume.

Figure 2 | Lysis Buffer Effects

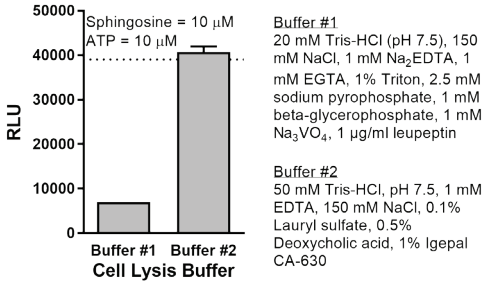
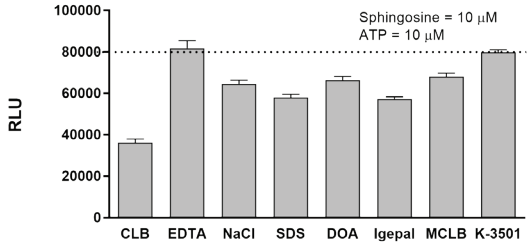


Figure 3 I Lysis Buffer Effects



Assay Protocol

Please read this entire section and assay notes before beginning .

1. Bring the assay kit to room temperature before use.
2. Add 50 µL deionized water into 50 µmol dried DTT (K-DTT1). Vortex to mix. This is the 1 M DTT stock. The 1 M DTT stock is stable for at least 1 month when stored at -20°C.
3. Supplement reaction buffer (K-3501) with 1 mM DTT prior to use. Dilulte 1 M DTT stock 1:1000 with reaction buffer (K-3501). Keep the complete reaction buffer on ice. Dilute only what is needed. The diluted DTT in reaction buffer is not stable.
4. Prepare a 4X sphingosine solution by diluting the 10 mM sphingosine stock (K-4401) in the complete reaction buffer (step 3). Keep on ice. Suggested sphingosine concentration – 100 µM (4X at 400 µM).
5. Prepare a 2X ATP solution by diluting the 10 mM ATP stock (K-ATP1) in the complete reaction buffer (step 3). Keep on ice. Suggested ATP concentration – 10 µM (2X at 20 µM).
6. Prepare 2-fold series ATP standards by diluting 10 mM ATP stock (K-ATP1) in 0.5 mL centrifuge tubes. Sample of a 10 µM ATP 2X dilution standard curve is shown on the following page (Table 1).
7. Add 10 µL of 4X sphingosine solution (step 4) per well. Then, add 10 µL of samples per well. For the ATP standard wells, add 10 µL of the complete reaction buffer (step 5) or sample buffer. Suggested plate layout in duplicates is on the following page (Table 2).
8. Start the reaction by adding 20 µL of the 2X ATP solution (step 5) to the sample wells. Add 20 µL of the 2-fold ATP dilution series (step 6) to the standard wells. Cover with plate seal. Mix by tapping the plate or by plate shaker. Incubate the reaction at room temperature. Incubation times may vary depending on

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the SPHK activity within samples. Suggested incubation time: 30 minutes to 2 hours.

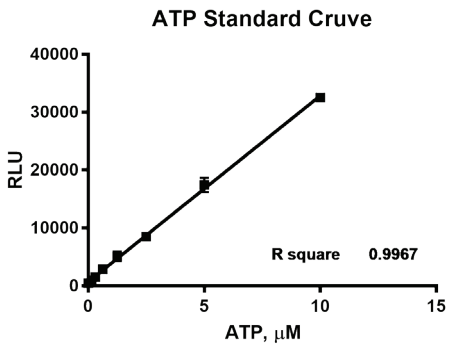
9. After incubation, add 40 µL of ATP Detector (K-LUMa) per well. Tap the plate or use a plate shaker to mix. Incubate at room temperature for at least 10 minutes to stabilize the luminescent signal. Protect from light.
10. Record luminescence.

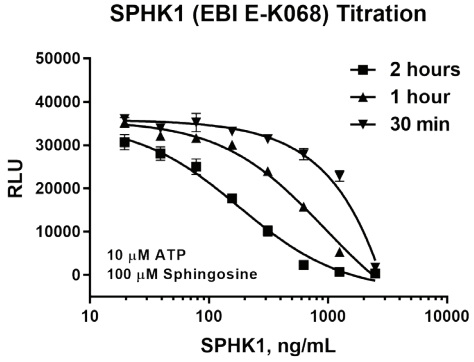
Table 1, ATP Standards

Final ATP Conc. in Reaction (1X)	2X ATP Solutions	ATP stock (K-ATP1) or previous dilution	Complete Reaction Buffer (step 3)
10 µM	20 µM	1 µL of K-ATP1	499 µL
5 µM	10 µM	100 µL of 20µM soln.	100 µL
2.5 µM	5 µM	100 µL of 10µM soln.	100 µL
1.25 µM	2.5 µM	100 µL of 5µM soln.	100 µL
0.625 µM	1.25 µM	100 µL of 2.5µM soln.	100 µL
0.3125 µM	0.625 µM	100 µL of 1.25µM soln.	100 µL
0.15625 µM	0.3125 µM	100 µL of 0.625µM soln.	100 µL
0 µM	0 µM	---	100 µL

Table 2, Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	10 µM ATP	10 µM ATP	*Sample 1*	*Sample 1*	*Sample 9*	*Sample 9*	*Sample 17*	*Sample 17*	*Sample 25*	*Sample 25*	*Sample 33*	*Sample 33*
B	5 µM ATP	5 µM ATP	*Sample 2*	*Sample 2*	*Sample 10*	*Sample 10*	*Sample 18*	*Sample 18*	*Sample 26*	*Sample 26*	*Sample 34*	*Sample 34*
C	2.5 µM ATP	2.5 µM ATP	*Sample 3*	*Sample 3*	*Sample 11*	*Sample 11*	*Sample 19*	*Sample 19*	*Sample 27*	*Sample 27*	*Sample 35*	*Sample 35*
D	1.25 µM ATP	1.25 µM ATP	*Sample 4*	*Sample 4*	*Sample 12*	*Sample 12*	*Sample 20*	*Sample 20*	*Sample 28*	*Sample 28*	*Sample 36*	*Sample 36*
E	0.63 µM ATP	0.63 µM ATP	*Sample 5*	*Sample 5*	*Sample 13*	*Sample 13*	*Sample 21*	*Sample 21*	*Sample 29*	*Sample 29*	*Sample 37*	*Sample 37*
F	0.31 µM ATP	0.31 µM ATP	*Sample 6*	*Sample 6*	*Sample 14*	*Sample 14*	*Sample 22*	*Sample 22*	*Sample 30*	*Sample 30*	*Sample 38*	*Sample 38*
G	0.16 µM ATP	0.16 µM ATP	*Sample 7*	*Sample 7*	*Sample 15*	*Sample 15*	*Sample 23*	*Sample 23*	*Sample 31*	*Sample 31*	*Sample 39*	*Sample 39*
H	0 µM ATP	0 µM ATP	*Sample 8*	*Sample 8*	*Sample 16*	*Sample 16*	*Sample 24*	*Sample 24*	*Sample 32*	*Sample 32*	*Sample 40*	*Sample 40*





Data Analysis

Sphingosine kinase activity can be estimated by comparing the luminescence values from the wells containing enzyme reaction products to the values in the ATP standard curve. Plot the luminescence values obtained in the ATP standard curve vs concentration of ATP to generate a standard curve using linear regression analysis. Determine the level of ATP in each of your reaction wells in µM by interpolation from the luminescence values obtained from the enzyme reactions. Sphingosine Kinase activity in your samples can also be estimated by % conversion from the initial ATP amount (10 µM ATP) once the reaction wells have been interpolated.

Calculate % signal reduction (or % conversion) using the formula below:

$$\text{Signal reduction} = 1 - \left(\frac{\text{(RLU sample)}}{\text{(RLU 10 µM ATP)}} \right) \times 100 = \_\_\_\_\_$$

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References

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Related Products

Catalog #	Products
B-0024	Sphingosine Kinase Inhibitor
B-0720	FTY720 (Sphingosine Analog)
E-K068	Sphingosine Kinase 1, active
E-K069	Sphingosine Kinase 2, active
K-1900	Sphingosine 1 Phosphate (S1P) ELISA Kit
S-1000	Sphingosine
S-100B	Sphingosine-biotin
S-100F	Sphingosine-fluorescein
S-100T	Sphingosine-tetramethylrhodamine
S-6100	SphingoBeads

Please visit our website at www.echelon-inc.com for more enzyme and lipid products.

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