

An Improved Isoform-Selective Assay for Sphingosine Kinase 1 Activity

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Abstract

Sphingosine kinases (SK) are the sole enzymes responsible for the production of sphingosine 1-phosphate (S1P). S1P is a signaling molecule with a plethora of targets, acting as both a second messenger intracellularly and extracellularly via a family of cell surface G-protein-coupled S1P receptors. The two sphingosine kinases, SK1 and SK2, arise from different genes and have some distinct and overlapping cellular functions that are regulated in part by differential cellular localization, developmental expression, and catalytic properties. Here, we describe an improved method for selectively detecting SK1 activity in vitro and cell lysates via the use of the zwitterionic detergent CHAPS, which effectively inhibits SK2 activity and thus allows selective analysis of SK1 activity in a range of cell samples. The assay measures the production of ^{32}P -labeled S1P following the addition of exogenous sphingosine and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The S1P product can be purified by Bligh–Dyer solvent extraction, separated by thin layer chromatography (TLC), and the radiolabeled S1P quantified by exposing the TLC plate to a storage phosphor screen. This sensitive, reproducible assay can be used to selectively detect SK1 activity in tissue, cell, and recombinant protein samples.

Keywords: Sphingosine kinase, *D-erythro*-sphingosine, Sphingosine 1-phosphate, Thin layer chromatography, Bligh–Dyer extraction

1 Introduction

Sphingosine 1-phosphate (S1P) is a bioactive signaling molecule that can modulate a range of cellular responses. S1P can be exported from the cell to act as a ligand for a family of S1P-specific G-protein-coupled receptors or bind to and act on a range of intracellular targets [1, 2]. The production of S1P is catalyzed by two sphingosine kinase (SK) enzymes, SK1 and SK2, that phosphorylate sphingosine at the primary hydroxyl (Fig. 1). SK1 and SK2 are distinct proteins that arise from different genes. Knockout of both SK isoforms is embryonic lethal in mice; however, loss of only one isoform produces no obvious defects, suggesting some level of functional redundancy between these two enzymes [3–5]. Both SK1 and SK2 promote cell survival and proliferation [6, 7], although, in some cases SK2 can exert different physiological functions. As opposed to SK1, SK2 can have proapoptotic effects [8–10], inducing epigenetic and transcriptional regulation by acting on histone deacetylases

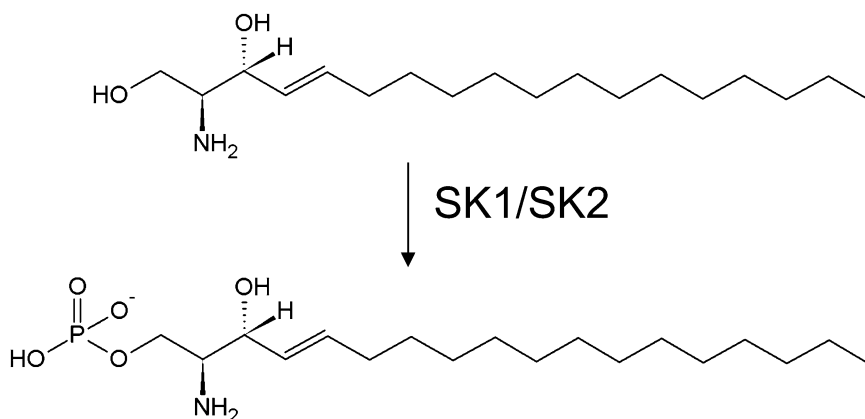


Fig. 1 Sphingosine kinases catalyze the phosphorylation of sphingosine to sphingosine 1-phosphate (S1P)

[11]. In a number of conditions, SK1 and SK2 appear to play distinct roles, including in inflammatory arthritis [12], ischemia-reperfusion injury [13], lipopolysaccharide-induced lung injury [14], in human mast cell functions [15], and S1P distribution in blood and lymphoid tissue [16].

The two SK enzymes share significant sequence similarity; however, they differ in size, post-translational modifications, cellular localization, and expression profile [17]. In particular, SK1 and SK2 differ in catalytic properties, a factor that can be exploited to selectively measure activity of individual isoforms [18, 19]. The most commonly employed and cost-effective SK assay measures the production of ³²P-labeled S1P from sphingosine and [γ -³²P]ATP [20, 21]. Conditions in the assay can be tailored to selectively measure the activity of SK1, SK2, or both. Previously, we and others have reported conditions for the selective assay of SK1 activity using the addition of 0.25 % Triton X-100 to assays, with this detergent largely inhibiting SK2 activity but sustaining SK1 activity when assessed with purified recombinant SK proteins [18, 19]. Unexpectedly, however, we recently found that these previously described SK1-selective assay conditions were not very selective for SK1 when employed with cell lysates. Indeed, using these conditions, cell lysates from embryonic fibroblasts (MEFs) from SK1 knockout mice (which only have the SK2 isoform) showed significant residual SK2 activity (Fig. 2a). Higher concentrations of Triton X-100 also failed to improve SK2 inhibition in lysates (Fig. 2b). Therefore, we tested the ability of other detergents to inhibit purified recombinant SK2 and found that at a concentration of 1 % the zwitterionic detergent CHAPS effectively eliminated SK2 activity (Fig. 3a), albeit with a substantial (3.5-fold) increase in the activity of purified recombinant SK1. At 3 % CHAPS, the activity of SK2 was negligible and the increase in SK1 activity was less than twofold (Fig. 3b). Importantly, when SK2 activity was determined in lysates from SK1 knockout MEFs (which only have the SK2 isoform), the addition of 3 % CHAPS completely inhibited SK2

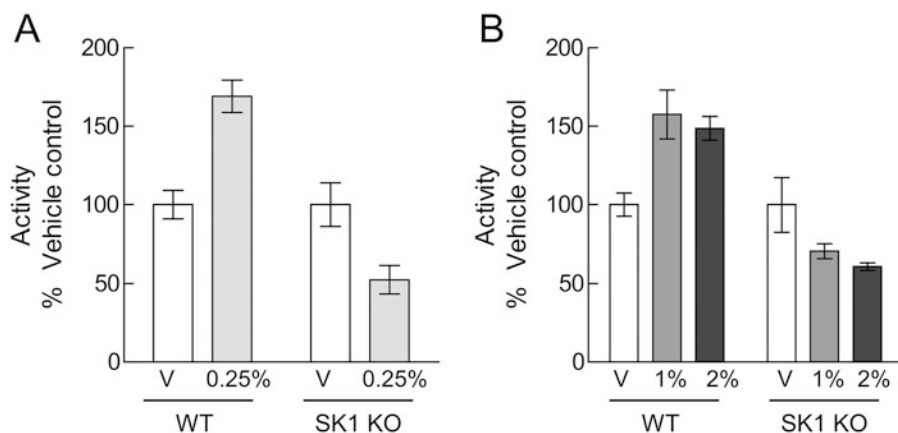


Fig. 2 Triton X-100 does not adequately inhibit SK2 activity in lysates. (a) Lysates from wild-type (WT) mouse embryonic fibroblasts (MEFs) (*open bars*) and SK1 knockout (KO) MEFs (*grey bars*) were assayed using non-selective SK assay conditions with either H₂O vehicle (V), as well as 0.25 % Triton X-100 to test SK2 inhibition. (b) Triton X-100 was tested at both 1 % (*grey*) and 2 % (*dark grey*) in WT and SK1 KO MEF lysates. Activities are standardized to 100 % of vehicle control for the WT and SK1 KO MEFs and correspond to 618 and 158 pmol/mg/min specific activities, respectively. Data represent the mean $\pm$ S.D. of four independent experiments

activity, and modestly elevated SK1 activity (1.3-fold) in lysates from MEFs of wild-type mice (Fig. 4a). This 1.3-fold increase in SK1 activity was consistent with SK1 activity measured in lysates from MEFs of SK2 knockout mice treated with 3 % CHAPS versus vehicle (data not shown). Hence, inclusion of CHAPS in the assay significantly improves the stringency of the SK1 selectivity. Assays employing BSA-solubilized sphingosine without addition of CHAPS can be used in parallel to measure the total SK activity (from both SK1 and SK2) in cell lysates.

At the completion of the assay incubation, the S1P product is isolated using a Bligh–Dyer solvent extraction, where under acidic conditions around 75 % of the S1P partitions to the organic phase [20]. The chloroform phase is then separated by thin layer chromatography (TLC) where the radiolabeled S1P product migrates with an R_f of 0.7 in butanol/ethanol/H₂O/glacial acetic acid (8:2:1:2) (Fig. 4b). The S1P spot can then be quantified by exposing the TLC plate to a storage phosphor screen.

2 Materials

Prepare all solutions with analytical grade reagents using distilled water, and store at room temperature unless otherwise indicated.

2.1 Sample Preparation

1. Lysis buffer: 50 mM Tris/HCl buffer (pH 7.4) containing 150 mM NaCl, 10 % glycerol (w/v), 1 mM dithiothreitol, 2 mM Na₃VO₄, 10 mM NaF, 10 mM β -glycerophosphate, and 1 mM EDTA (*see Note 1*)

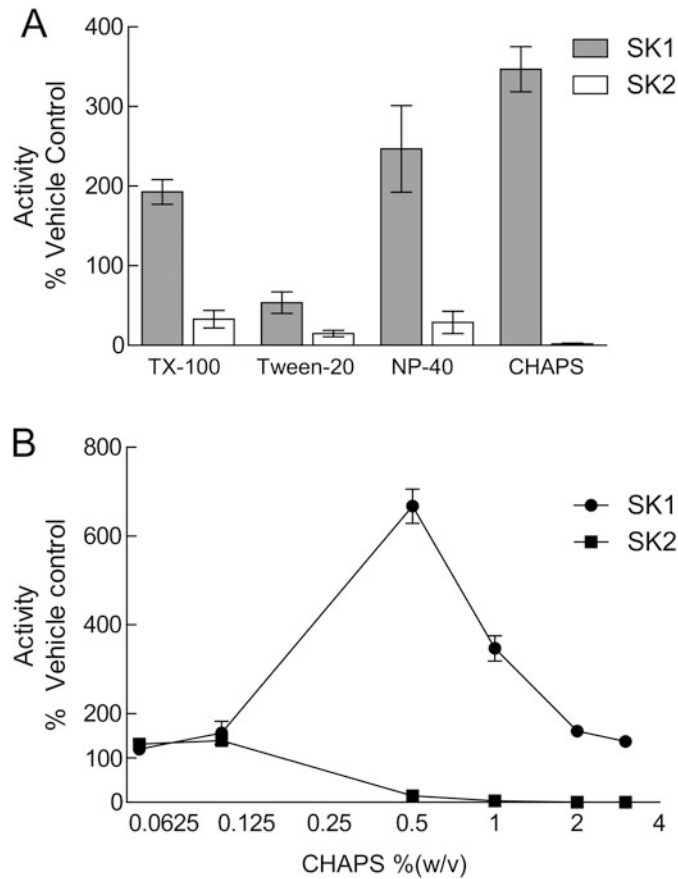


Fig. 3 Effect of other detergents on the activity of SK1 and SK2. **(a)** Purified recombinant human SK1 (*grey bars*) and SK2 (*open bars*) were assayed under nonselective SK assay conditions with the addition of H₂O vehicle or 1 % (w/v, final) detergent **(b)** varying concentrations of CHAPS were tested against recombinant purified SK1 (*circles*) and SK2 (*squares*). Activity of SK1 and SK2 with vehicle was used as the controls, and set as 100 %. Data represent the mean $\pm$ S.D. of three independent experiments

2. Protease inhibitor cocktail (CompleteTM; Roche) prepared as a 50 $\times$ concentrated stock and stored at -20°C
3. Bath sonicator with sufficient power to disrupt cells (e.g., BioruptorTM; Diagenode, NY) or 26G needle and syringe
4. Microcentrifuge

2.2 Substrate Preparation

1. Fatty acid-free BSA
2. Sonicator

2.3 Incubation

1. Eppendorf[®] Safe-Lock[®] 1.5 mL microcentrifuge tubes (*see Note 2*)
2. SK1-selective assay buffer: 100 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM Na₃VO₄, 10 mM NaF, and 4.4 % (w/v) CHAPS

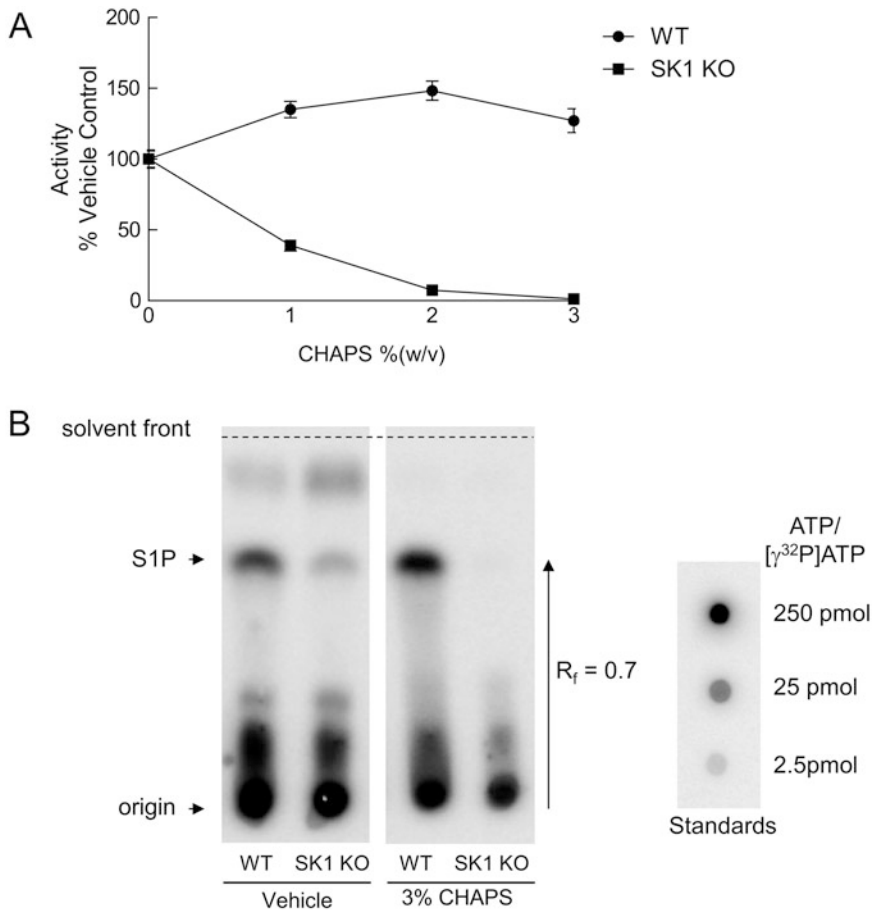


Fig. 4 3 % CHAPS inhibits SK2 activity in cell lysates. **(a)** Lysates from wild-type (WT) MEFs (*circles*) and SK1 knockout (KO) MEFs (*squares*) were assayed using nonselective SK assay conditions with either H_2O vehicle (V), as well as 1–3 % CHAPS to test SK2 inhibition. Activities are standardized to 100 % of vehicle control for the WT and SK1 KO MEFs and correspond to 618 and 158 pmol/mg/min specific activities, respectively. Data represent the mean $\pm$ S.D. of four independent experiments. **(b)** Image of TLC plates with S1P spots from nonselective (vehicle) and SK1 selective (3 % CHAPS) assays using lysates from WT or SK1 KO MEFs (using 25 μg of protein, 2 μCi [$\gamma^{32}\text{P}$]ATP, and 0.5 mM adenosine-5'-triphosphate (ATP)). The chloroform phase was spotted onto the plate at the origin and using the described mobile phase S1P migrates with an (R_f) of 0.7. The S1P can be quantified and converted to pmol S1P generated by comparison to standards made from dilution of the assay reaction mixture 1 in 10, 1 in 100, and 1 in 1,000 to allow application of 250, 25, and 2.5 pmol of [$\gamma^{32}\text{P}$]ATP, respectively (for standard assay conditions with 1 μCi [$\gamma^{32}\text{P}$]ATP outlined in Table 1, with 2 μCi [$\gamma^{32}\text{P}$]ATP values are 500, 50, and 5 pmol)

3. Total SK assay buffer: 100 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM Na_3VO_4 , and 10 mM NaF
4. D-*erythro*-sphingosine (Biomol)
5. 20 mM Adenosine-5'-triphosphate (ATP) prepared in 1 M Tris/HCl buffer (pH 7.4) containing 200 mM MgCl_2 and stored at -20°C (*see* **Note 3**)

6. [$\gamma^{32}\text{P}$]ATP (Perkin Elmer, NEG502A)
7. 50 mM 4-deoxypyridoxine (Sigma, DO501) stock solution stored at $-20\text{ }^{\circ}\text{C}$ (*see* **Note 4**)
8. Perspex screens for use with radionuclides
9. Geiger counter
10. Water bath or incubator set at $37\text{ }^{\circ}\text{C}$
11. Whatman paper (3 M)

2.4 Extraction

1. Chloroform/methanol/conc. HCl (100:200:1)
2. Chloroform
3. 5 M KCl
4. Microcentrifuge
5. Aspiration apparatus
6. Silica gel TLC plates (Sigma, Z193291, pore size $60\text{ }\text{\AA}$, aluminum backing)

2.5 Resolution and Quantitation of Sphingosine 1-Phosphate

1. Source of compressed air or hair dryer (on cool setting)
2. 1-Butanol/ethanol/glacial acetic acid/ H_2O (8:2:1:2)
3. Glass TLC developing tank
4. Plastic ziplock bags or cling wrap
5. Storage phosphor screen (GE Healthcare)
6. Phosphorimaging system (e.g., Typhoon; GE Healthcare)
7. ImageQuantTM (GE Healthcare) software or equivalent

3 Methods

Ensure all procedures using [$\gamma^{32}\text{P}$]ATP are carried out with protective perspex shielding using standard radiation safety techniques. Radiation should be monitored with a Geiger counter. Dispose of all waste strictly in accordance with local radioactive waste disposal regulations.

3.1 Sample Preparation

1. Resuspend cell pellets in lysis buffer.
2. Lyse by sonication in a bath sonicator (e.g., $4 \times 25\text{ s}$ pulses with 25 s breaks in a 200 W BioruptorTM apparatus) or by five passages through a 26G needle.
3. Samples can be assayed as whole cell lysates, or clarified lysate can be prepared by centrifuging the whole cell lysate at $13,000 \times g$ for 15 min at $4\text{ }^{\circ}\text{C}$.
4. Assess protein concentration in the samples by standard protein assay methods.

3.2 Preparing the Sphingosine Substrate

1. Weigh out the desired quantity of sphingosine to make up 2 mM sphingosine in the required volume.
2. Add 2 % fatty acid-free BSA in 50 mM Tris/HCl (pH 7.4).
3. Sonicate on ice until the solution becomes clear.
4. Aliquot and store at -20°C (*see* **Note 5**).

3.3 Incubation

1. Add 20 μl of enzyme sample to an Eppendorf[®] Safe-Lock[®] microcentrifuge tube (*see* **Note 6**).
2. Add 80 μl of reaction mixture, the composition of which differs depending on the SK isoform to be analyzed (*see* **Table 1** and **Notes 7** and **8**).
3. Incubate at 37°C for 30 min.
4. For later conversion of radioactive signal to phosphate concentration, perform a tenfold serial dilution of the leftover reaction mixture into water and spot 2 μl of the 1 in 10, 1 in 100, and 1 in 1,000 dilutions onto pre-marked Whatman paper (*see* **Note 9**).

3.4 Extraction

1. To the 100 μl assay mixture, add 270 μl of chloroform/methanol/conc. HCl (100:200:1).
2. Add 20 μl of 5 M KCl (*see* **Note 10**).
3. Add 70 μl chloroform to create a phase separation.
4. Vortex to mix well.

Table 1
Example of assay setup

Reagent	Volume per assay (μl)	
	SK1	SK1 and SK2
20 mM Mg-ATP	5	5
10 $\mu\text{Ci}/\mu\text{l}$ [$\gamma^{32}\text{P}$]ATP	0.1	0.1
2 mM sphingosine in 2 % fatty acid-free BSA	5	5
50 mM 4-deoxypyridoxine	1	1
Assay buffer with 4.4 % (w/v) CHAPS	69	0
Assay buffer	0	69
Cell lysate or recombinant SK protein	20	20
Total volume	100	100

For selective detection of SK1 activity, use 3 % CHAPS to inhibit SK2. To nonselectively assay total SK activity, use BSA-solubilized sphingosine and omit CHAPs from the assay. It is recommended to prepare a batch of reaction mixture of sufficient volume to assay all of the required samples. To do this, we recommend multiplying each of the volumes shown by $n + 1$, where n is the number of samples to be assayed

5. Centrifuge for 5 min at $13,000 \times g$ to fully separate the phases (*see Note 11*).
6. Remove the upper aqueous/methanol phase by aspiration.

3.5 Thin Layer Chromatography

1. Cut a 20 cm $\times$ 20 cm Silica TLC plate into half (*see Note 12*).
2. Measure 2 cm from the bottom of the TLC plate and draw a line with pencil along the long edge.
3. Mark on this line with pencil where the samples will be applied (the origin), ensuring that the spots are no closer than 1.5 cm from the edge of the plate and no closer than 1.3 cm to each other (*see Note 13*).
4. Apply 50 μ l of the remaining lower chloroform phase onto the TLC plate by slowly and repeatedly spotting a few microliters of liquid onto the plate with the pipette tip so that no more than a 5 mm diameter circle of the TLC plate is wet (*see Note 14*). Between each spot, allow the liquid to absorb into the plate and then carefully dry the spot with a stream of air.

3.6 Resolution and Quantitation of Sphingosine 1-Phosphate (See Note 15)

1. Develop the TLC plate with 1-butanol/ethanol/glacial acetic acid/ H_2O (8:2:1:2) in a glass TLC developing tank until the mobile phase is within 1 cm of the top of the TLC plate (*see Note 16*).
2. Remove the TLC plate from the developing tank with tweezers and allow to air dry in a fume hood for 15 min.
3. Cover the dried TLC plate in cling wrap or a place in a ziplock plastic bag.
4. Expose to a storage phosphor screen overnight (*see Note 17*). Include the Whatman paper with the assay mixture dilutions to allow quantification of the phosphor signal.
5. Read the storage phosphor screen on a phosphorimager.
6. Quantify the S1P spot which with the mobile phase employed will have a relative migration (R_f) of approximately 0.7 (*see Fig. 4*).
7. Using the included [$\gamma^{32}P$]ATP standard curve, the sample protein concentrations, and a multiplication coefficient of 4.27 to account for incomplete S1P extraction and spotting of only part of the chloroform phase onto the TLC plate (*see Note 18*), the S1P spot intensity can be converted to amount of phosphate transferred/min/mg protein.

4 Notes

1. Since both SK1 and SK2 can be activated by phosphorylation [22, 23], inclusion of phosphatase inhibitors NaF, Na_3VO_4 , and β -glycerophosphate in the lysis buffer is important.

Activation of Na_3VO_4 is required prior to its use to enable phosphatase inhibition [24].

2. The Eppendorf® Safe-Lock® microcentrifuge tubes have a better sealing cap that contains the radioactive aerosols during centrifugation steps more effectively than regular microcentrifuge tubes. If other microcentrifuge tubes are employed, the tops of the closed tubes should be covered with Parafilm™ to minimize potential microcentrifuge contamination by radionuclides.
3. ATP must be in complex with Mg to be a substrate for phosphotransferases. The ATP solution must be prepared in a buffer to prevent the solution becoming too acidic which will result in ATP hydrolysis.
4. 4-Deoxypyridoxine is an inhibitor of S1P lyase and is only required for use when assaying SK activity in cell lysates to prevent dephosphorylation of the S1P product.
5. This solution should be aliquoted but can tolerate several freeze-thaw cycles.
6. **Cell lysates:** 20–50 μg of cell lysates are generally used for the assay. SK1 selectivity with >50 μg lysate would need to be tested empirically. **Recombinant Protein:** For purified recombinant SK proteins it is recommended to use between 0.1 and 10 ng of recombinant protein per assay. Too much enzyme will cause the substrates to become limiting, causing a nonlinear reaction rate. It is advisable to initially do a time course, stopping the assay at 10, 20, and 30 min timepoints to check that the substrates are not limiting.
7. The CHAPS concentration in the reaction mixture is $1.47\times$ to account for the 1.47-fold dilution by the 20 μl sample and substrates/additives. It is recommended to prepare a batch of reaction mixture of sufficient volume to assay all of the required samples (see Table 1). The following gives the assay components and their final concentrations in each of the assays:
SK1-selective assay: 1 mM Mg-ATP, 1 μCi [$\gamma^{32}\text{P}$]ATP, 100 μM sphingosine in 0.1 % fatty acid-free BSA (prepared from 2 mM sphingosine stock in 2 % fatty acid-free BSA), 0.5 mM 4-deoxypyridoxine, and assay buffer with 3 % (w/v, final) CHAPS
Nonselective SK assay: 1 mM Mg-ATP, 1 μCi [$\gamma^{32}\text{P}$]ATP, 100 μM sphingosine in 0.1 % fatty acid-free BSA (prepared from 2 mM sphingosine stock in 2 % fatty acid-free BSA), 0.5 mM 4-deoxypyridoxine, and CHAPS-free assay buffer
8. If enhanced assay sensitivity is required the amount of [$\gamma^{32}\text{P}$]ATP added to the assay can be increased twofold, and the concentration of unlabeled ATP can be decreased to 0.5 mM. Lower ATP concentrations than this should not be routinely

used to ensure that ATP does not limit the reaction velocity since the K_M value for ATP of SKI is approximately 80 μM [25].

9. Using standard assay conditions (Table 1), 2 μl of reaction mixture diluted 10, 100, and 1,000-fold will contain 250, 25, and 2.5 pmol of $[\gamma^{32}\text{P}]\text{ATP}$, respectively (Fig. 4).
10. Addition of KCl enhances S1P extraction into the organic (chloroform) phase [26].
11. In cell lysates, this step often results in the appearance of a “skin” between the two phases that consists mostly of precipitated proteins.
12. The solvent system described here effectively separates S1P from other radiolabeled contaminants in the chloroform phase within 9 cm migration of the mobile phase from the origin. Thus, TLC plates of 10 cm height are sufficient.
13. It is recommended that a maximum of 14 samples be spotted per 20 cm TLC plate for optimum results. Plates can be cut down further for smaller assays.
14. Rinse pipette tips with chloroform prior to transfer of the chloroform phase to minimize dripping of the sample from the pipette tip.
15. Solvent preparation and development of the TLC plates should be carried out in a fume hood.
16. This mobile phase will take approximately 1.5 h to migrate up the TLC plate. We find this mobile phase to be the most effective at resolving the S1P spot, but a number of other mobile phases have also been employed in this assay. These include: 1-butanol/glacial acetic acid/ H_2O (3:1:1) [26], methanol/chloroform/glacial acetic acid/ H_2O (10:4:3:2:1) [27], and chloroform/methanol/ammonium hydroxide (4:1:0.1, v/v) [20].
17. Storage phosphor screens capture images produced by ionizing radiation, such as ^{32}P . During reading, the phosphorimager stimulates the screen using lasers to convert the latent signal to light. This light is proportional to the amount of radioactivity in the sample. The image can then be quantified using standard quantification software such as ImageQuant™. The screens are reusable and can be cleared by exposure to extra-bright light using a light box. If a phosphorimager is not available, X-ray film can be employed, but this can be ten- to 100-fold less sensitive than the storage phosphor screen/phosphorimager approach.
18. Values calculated from the standard curve must be adjusted by a factor of 4.27 to account for the incomplete extraction of only 75 % of the generated S1P into the chloroform phase [21], and application of only 31.5 % of the chloroform phase onto the TLC plate.

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Sphingosine-1-Phosphate

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