

# Chapter 2

## Isoform-Selective Assays for Sphingosine Kinase Activity

Melissa R. Pitman, Duyen H. Pham, and Stuart M. Pitson

### Abstract

Sphingosine kinases (SK) 1 and 2 are unique lipid kinases that phosphorylate sphingosine to form sphingosine-1-phosphate (S1P). S1P is a bioactive molecule eliciting multiple effects both extracellularly via cell surface S1P receptors and intracellularly through a number of recently identified protein targets. The two enzymes arise from different genes, and differ in their cellular localisation, developmental expression, catalytic properties, and in at least some functional roles. Here, we describe methods for selectively detecting SK1 and SK2 activities *in vitro*, highlighting conditions that can discriminate between the activities of these two enzymes. The assays measure the production of  $^{32}\text{P}$ -labelled S1P following the addition of exogenous sphingosine and [ $\gamma^{32}\text{P}$ ] adenosine-5'-triphosphate. The S1P product can be purified by Bligh–Dyer solvent extraction, separated by thin-layer chromatography (TLC), and the radiolabelled S1P quantified by exposing the TLC plate to a storage phosphor screen. This sensitive, reproducible assay can be used to selectively detect SK1 and SK2 activities in tissue, cell, and recombinant protein samples.

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

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### 1. Introduction

Sphingosine-1-phosphate (S1P) mediates many cellular responses via its actions as both an intracellular second messenger and as a ligand for a family of five S1P-specific G protein-coupled receptors (1). The sole physiological route of S1P generation in mammalian cells is via the activities of two sphingosine kinases (SK1 and SK2) that transfer the  $\gamma$  phosphate of adenosine-5'-triphosphate (ATP) to the primary hydroxyl of sphingosine (Fig. 1).

SK1 and SK2 arise from different genes, and although they share significant sequence similarity, they differ in size, catalytic properties, cellular localisation, and expression profile (2–4).

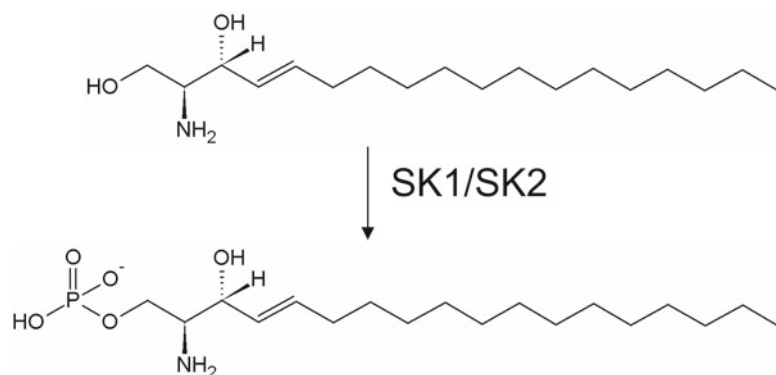


Fig. 1. Sphingosine kinases catalyse the phosphorylation of sphingosine to sphingosine-1-phosphate.

While mice lacking both SK1 and SK2 die in utero, loss of either individual SK isoform results in mice with no obvious defects, suggesting that at least some functional redundancy exists between these enzymes (5–7). Other studies, however, suggest that the two enzymes may have some quite different physiological functions. Indeed, SK1 and SK2 appear to play contrasting roles in a number of conditions, including inflammatory arthritis (8), ischemia–reperfusion injury (9) and lipopolysaccharide-induced lung injury (10), and also in human mast cell functions (11). Interestingly, SK1 is widely reported to promote cell survival and proliferation, while at least in some cases SK2 can have proapoptotic effects (12–14).

Since SK1 and SK2 appear to serve at least some distinct functions, it is important to be able to discriminate between their activities in samples. The most commonly employed SK assay measures the production of <sup>32</sup>P-labelled S1P from sphingosine and [ $\gamma$ -<sup>32</sup>P] ATP (15, 16). By performing this assay under different conditions (3), the activities of SK1 and SK2 can be selectively determined (Fig. 2). For example, SK1 is inhibited by 1 M KCl while SK2 retains catalytic activity under these conditions (Fig. 3a). Hence, including 1 M KCl in the SK assay allows for selective detection of SK2 activity. Furthermore, SK2 is inhibited by Triton X-100 concentrations of 0.1% (1.6 mM) and above (Fig. 3b), while the activity of SK1 is not adversely affected by this detergent, except at very high concentrations (Fig. 3b), where surface dilution of sphingosine in the Triton X-100 micelles is likely to occur (17). Hence, SK1 activity can be selectively determined via the use of Triton X-100 in the assay. Since Triton X-100 is commonly employed to solubilise sphingosine, SK2 activity must, instead, be measured with sphingosine solubilised with bovine serum albumin (BSA). Assays employing BSA-solubilised sphingosine without addition of Triton X-100 or KCl can be used to measure the total SK activity (from both SK1 and SK2) in cell lysates.

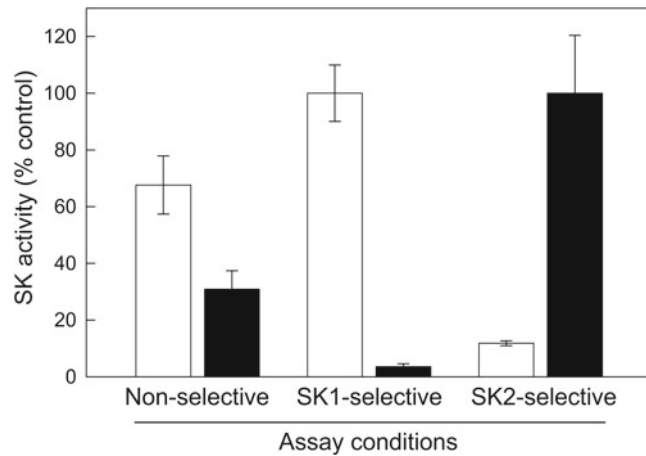


Fig. 2. Selective conditions for the determination of SK1 and SK2 activities. Purified recombinant human SK1 (*open bars*) and SK2 (*filled bars*) were assayed using non-selective SK assay conditions, as well as SK1- and SK2-selective assay conditions. Activities from SK1- and SK2-selective conditions were used as the controls for SK1 and SK2 activities, respectively, and set as 100%. Data represent the mean  $\pm$  S.D. of three independent experiments.

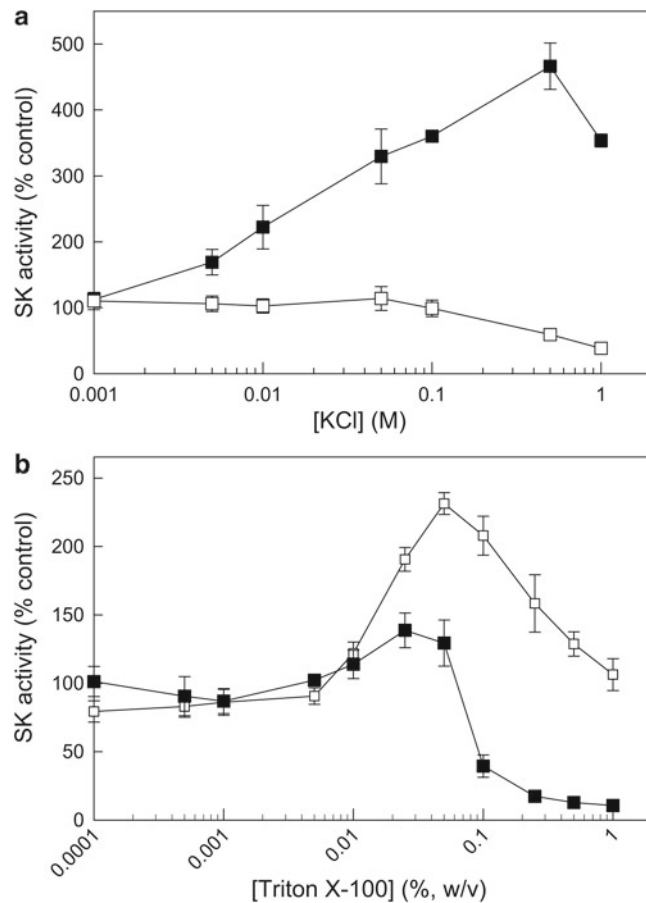


Fig. 3. Effect of Triton X-100 and KCl on the activities of SK1 and SK2. Purified recombinant human SK1 (*open squares*) and SK2 (*filled squares*) were assayed under non-selective SK assay conditions, but with the addition of varying concentrations of either (**a**) KCl or (**b**) Triton X-100. Activities of SK1 and SK2 in the absence of KCl and Triton X-100 were used as the controls, and set as 100%. Data represent the mean  $\pm$  S.D. of three independent experiments.

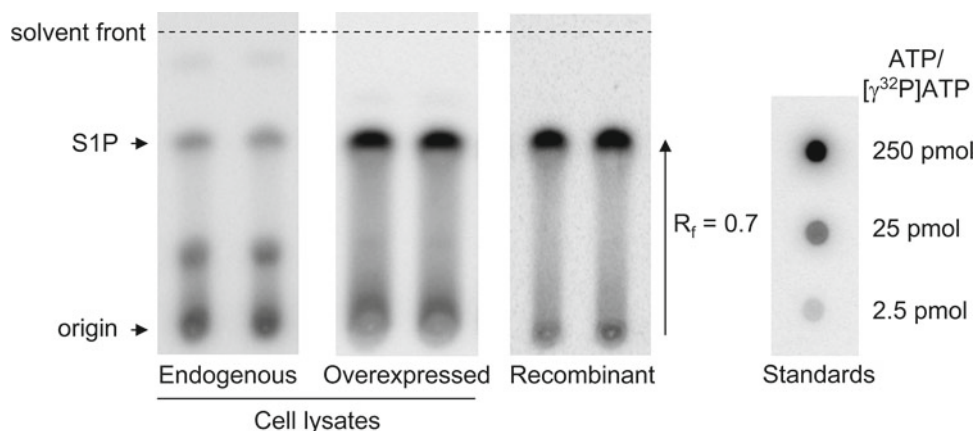


Fig. 4. Examples of sphingosine kinase assays. Image of TLC plates with S1P spots from SK1 assays using lysates from untransfected cells (measuring endogenous SK1 activity), cells overexpressing SK1, and purified recombinant SK1. The chloroform phase is spotted onto the plate at the origin and using the described mobile-phase S1P migrates with an ( $R_f$ ) of 0.7. The S1P can be quantified and converted to picomole 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 ATP/[ $\gamma^{32}\text{P}$ ]ATP, respectively.

Once the enzyme reaction is complete, the S1P product is purified by Bligh–Dyer solvent extraction, where under acidic conditions around 75% of the S1P partitions to the organic phase (15). The S1P in this chloroform phase is then separated by thin-layer chromatography (TLC), where S1P migrates with an  $R_f$  of 0.7 in butanol/ethanol/glacial acetic acid/ $\text{H}_2\text{O}$  (8:2:1:2) (Fig. 4). The radiolabelled S1P 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), 0.05% (w/v) Triton X-100, 1 mM dithiothreitol, 2 mM  $\text{Na}_3\text{VO}_4$ , 10 mM NaF, 10 mM  $\beta$ -glycerophosphate, 1 mM EDTA (see Note 1).
2. Protease inhibitor cocktail (Complete™; Roche) prepared as a 50× concentrated stock and stored at  $-20^\circ\text{C}$ .

3. Bath sonicator with sufficient power to disrupt cells (e.g. Bioruptor™; Diagenode, NY) or 26G needle and syringe.
4. Microcentrifuge.

## **2.2. Substrate Preparation**

1. Fatty acid-free BSA.
2. 5% (w/v) Triton X-100.
3. Sonicator.

## **2.3. Incubation**

1. Eppendorf® Safe-Lock® 1.5-mL microcentrifuge tubes (see Note 2).
2. SK1 assay buffer: 100 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF.
3. SK2 assay buffer: 100 mM Tris/HCl (pH 7.4), 1.4 M KCl, 150 mM NaCl, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 10 mM NaF (see Note 3).
4. D-*erythro*-sphingosine (Biomol).
5. 20 mM ATP prepared in 1 M Tris/HCl buffer (pH 7.4) containing 200 mM MgCl<sub>2</sub> and stored at -20°C (see Note 4).
6. [ $\gamma^{32}$ P]ATP (Perkin Elmer, NEG502A).
7. 50 mM 4-deoxypyridoxine (Sigma, DO501) stock solution stored at -20°C (see Note 5).
8. Perspex screens for use with radionuclides.
9. Geiger counter.
10. Water bath or incubator set at 37°C.
11. Whatman paper (3MM).

## **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 Å, aluminium backing).

## **2.5. Resolution and Quantitation of S1P**

1. Source of compressed air or hair dryer (on cool setting).
2. 1-Butanol/ethanol/glacial acetic acid/H<sub>2</sub>O (8:2:1:2).
3. Glass TLC developing tank.
4. Plastic zip-lock bags or cling wrap.
5. Storage phosphor screen (GE Healthcare).
6. Phosphorimaging system (e.g. Typhoon; GE Healthcare).
7. ImageQuant™ (GE Healthcare) software or equivalent.

### 3. Methods

Ensure that 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.  $4 \times 25$ -s pulses with 25-s breaks in a 200 W Bioruptor™ 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°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 either 5% Triton X-100 (w/v) or 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 6).

#### 3.3. Incubation

1. Add 20  $\mu\text{l}$  of enzyme sample to an Eppendorf® Safe-Lock® microcentrifuge tube (see Note 7).
2. Add 80  $\mu\text{l}$  of reaction mixture, the composition of which differs depending on the SK isoform to be analysed (see Table 1 and Notes 8 and 9).
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  $\mu\text{l}$  of the 1 in 10, 1 in 100, and 1 in 1,000 dilutions onto pre-marked Whatman paper (see Note 10).

#### 3.4. Extraction

1. To the 100  $\mu\text{l}$  assay mixture, add 270  $\mu\text{l}$  of chloroform/methanol/conc. HCl (100:200:1).
2. Add 20  $\mu\text{l}$  of 5 M KCl (see Note 11).
3. Add 70  $\mu\text{l}$  chloroform to create a phase separation.
4. Vortex to mix well.

**Table 1**  
**Example of assay set-up**

Reagent	Volume per assay (μl)		
	SK1	SK2	SK1 and SK2
20 mM Mg-ATP	5	5	5
10 μCi/μl [ $\gamma^{32}$ P]ATP	0.1	0.1	0.1
2 mM sphingosine in 5% Triton X-100	5	0	0
2 mM sphingosine in 2% fatty acid-free BSA	0	5	5
50 mM 4-deoxypyridoxine	1	1	1
Assay buffer	69	0	69
Assay buffer with 1.4 M KCl	0	69	0
Cell lysate or recombinant SK protein	20	20	20
<i>Total volume</i>	<i>100</i>	<i>100</i>	<i>100</i>

For selective detection of SK1 activity, use Triton X-100-solubilised sphingosine (to inhibit SK2). For selective detection of SK2 activity, use BSA-solubilised sphingosine and include 1 M KCl in the reaction (to inhibit SK1). To non-selectively assay SK activity, use BSA-solubilised sphingosine in the assay and prepare the sample in lysis buffer without Triton X-100. 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 12).
6. Remove the upper aqueous/methanol phase by aspiration.

### 3.5. Thin-Layer Chromatography

1. Cut a  $20 \times 20$ -cm Silica TLC plate in half (see Note 13).
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 14).
4. Apply 50 μl of the remaining lower chloroform phase onto the TLC plate by slowly and repeatedly spotting a few microlitres 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 15). 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 S1P (see Note 16)

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 17).

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 place in a zip-lock plastic bag.
4. Expose to a storage phosphor screen overnight (see Note 18). 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 [ $\gamma^{32}\text{P}$ ]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 19), the S1P spot intensity can be converted to the amount of phosphate transferred/min/mg protein.

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## 4. Notes

1. Since both SK1 and SK2 can be activated by phosphorylation (18, 19), inclusion of phosphatase inhibitors NaF,  $\text{Na}_3\text{VO}_4$ , and  $\beta$ -glycerophosphate in the lysis buffer is important. Activation of  $\text{Na}_3\text{VO}_4$  is required prior to its use to enable phosphatase inhibition (20). To measure SK2 activity in cell lysates, omit the Triton X-100 from the lysis buffer.
2. The Eppendorf<sup>®</sup> Safe-Lock<sup>®</sup> 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<sup>™</sup> to minimize potential microcentrifuge contamination by radionuclides.
3. For SK2-selective assays, prepare the assay buffer with 1.4 M KCl to give a final KCl concentration of 1 M in the assay.
4. 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.
5. 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.



6. This solution should be aliquoted but can tolerate several freeze–thaw cycles.
7. *Cell lysates*. 20  $\mu$ l of cell lysates are generally used for the assay, but this can be increased to 50  $\mu$ l if required to increase assay sensitivity when assaying samples with low SK activity. *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 non-linear reaction rate. It is advisable to initially do a time course, stopping the assay at 10-, 20-, and 30-min time points to check that the substrates are not limiting.
8. The concentrations in the reaction mixtures are 1.25 $\times$  these concentrations to account for the 1.25-fold dilution when added to the 20  $\mu$ l sample to be assayed. 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  $\mu$ Ci [ $\gamma^{32}$ P]ATP, 100  $\mu$ M sphingosine in 0.25% (w/v) Triton X-100 (prepared from 2 mM sphingosine stock in 5% Triton X-100), 0.5 mM 4-deoxypyridoxine, and assay buffer.

*SK2-selective assay*. 1 mM Mg-ATP, 1  $\mu$ Ci [ $\gamma^{32}$ P]ATP, 100  $\mu$ M sphingosine in 0.1% fatty acid-free BSA (prepared from 2 mM sphingosine stock in 2% fatty acid-free BSA), 1 M KCl, 0.5 mM 4-deoxypyridoxine, and assay buffer.

*Non-selective SK assay*. 1 mM Mg-ATP, 1  $\mu$ Ci [ $\gamma^{32}$ P]ATP, 100  $\mu$ M 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.
9. If enhanced assay sensitivity is required, the amount of [ $\gamma^{32}$ P]ATP added to the assay can be increased and the concentration of unlabelled 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 SK1 is approximately 80  $\mu$ M (2).
10. Using standard assay conditions, 2  $\mu$ l of reaction mixture diluted 10-, 100-, and 1,000-fold will contain 250, 25, and 2.5 pmol of [ $\gamma^{32}$ P]ATP, respectively (Fig. 4).
11. Addition of KCl enhances SIP extraction into the organic (chloroform) phase (21).
12. In cell lysates, this step often results in the appearance of a “skin” between the two phases that consists mostly of precipitated proteins.

13. The solvent system described here effectively separates S1P from other radiolabelled 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.
14. 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.
15. Rinse pipette tips with chloroform prior to transfer of the chloroform phase to minimise dripping of the sample from the pipette tip.
16. Solvent preparation and development of the TLC plates should be carried out in a fume hood.
17. 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<sub>2</sub>O (3:1:1) (21), methanol/chloroform/glacial acetic acid/H<sub>2</sub>O (10:4:3:2:1) (22), and chloroform/methanol/ammonium hydroxide (4:1:0.1, v/v) (15).
18. Storage phosphor screens capture images produced by ionizing radiation, such as <sup>32</sup>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 10–100-fold less sensitive than the storage phosphor screen/phosphorimager approach.
19. 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 (16) 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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