Sphingosine 1-Phosphate Assay Kit (S1P ELISA)

K-1900 (96 tests)

Support: echelon@echelon-inc.com

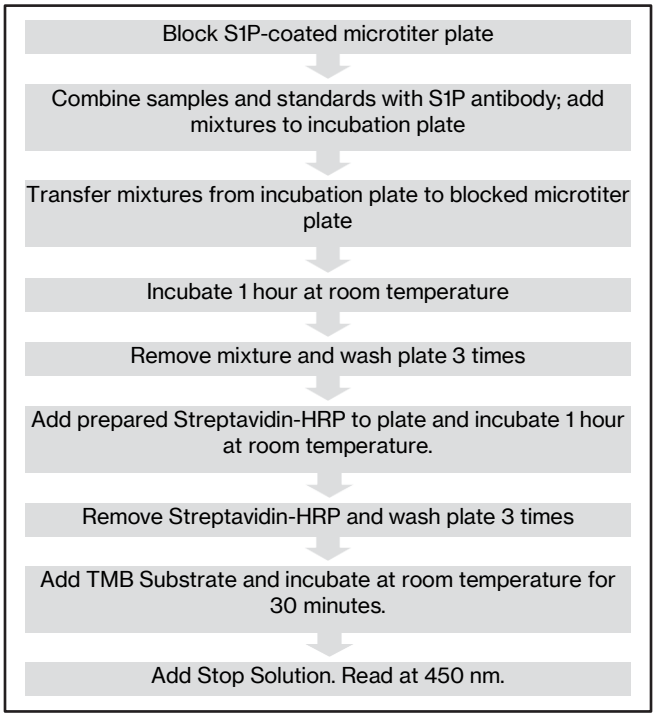
Description: The S1P ELISA is for the quantification of S1P in biological samples.

Storage: Store the S1P-ELISA kit at –20°C. Under proper storage conditions, the kit components are stable for at least 6 months from the date of receipt. Opened and reconstituted solutions are less stable.

Materials Provided

Catalog #	Description	Quantity
K-1901	Anti-S1P Antibody (Clone #LT1002)	Lyophilized
K-1903	Block Solution	30 mL
K-1904	Delipidized Serum (DHS)	15 mL
K-1905	Sphingosine 1-Phosphate (S1P) Standard	Lyophilized
K-1907	S1P Coated Microtiter Plate (12 X 8 strip-well plate)	1 Plate
K-PTAB	PBS Tablets	4 Tablets
K-PBST	Diluent Buffer	5 mL
K-SEC3h	Streptavidin HRP	80 µL
K-TMB1	TMB Substrate	12 mL
K-STOPt	1N Sulfuric Acid	8 mL
Mixing Plate	Natural 96-well polypropylene Plate	1 Plate
---	Plate Seals Clear Acetate Sheet, 1 side adhesive	2 Seals

Quick Protocol



Additional Materials Provided by User

- Pipettes (capable of delivering between 5 and 1,000 µL with appropriate tips)
- Multichannel pipettes
- Absorbance microplate reader capable of reading at 450 nm
- Plate Shaker (optional)

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Background

Sphingosine 1-Phosphate (S1P) is a crucial bioactive lipid mediator and key component of the sphingolipid signaling cascade. S1P, produced by the enzymes, Sphingosine Kinase 1 and 2 (SphK1, SphK2), initiates a proliferative[1], proangiogenic[2], and antiapoptotic[3] sequence of events that contributes to the progression of cancer, diabetes, and osteoporosis. SphK1 has been shown to be upregulated in a variety of cancers and recent research suggests that S1P itself, is a potent tumorigenic growth factor that may be a novel biomarker for early stage cancer detection[4,5]. S1P levels are tightly regulated by enzymes S1P Lyase (SPL), involved in its degradation, and two S1P-specific phosphatases and three lipid phosphatases that are involved in its synthesis. Additionally, S1P levels and its actions are controlled through a family of cell surface G protein receptors (S1PR1-5). These receptors have been implicated in a variety of developmental and disease related processes, such as immune[6] and vascular regulation[7]. S1P is also known for its direct role as a second messenger during inflammation[7] and SPhK/S1P/S1P-receptor signaling has been implicated in a variety of pathophysiological conditions and diseases such as atherosclerosis, cancer, diabetes, multiple sclerosis, sepsis, and so forth. Interference of these routes has the potential for the treatment of cancer, chronic inflammatory disorders and autoimmune diseases.

Assay Design

The S1P-ELISA is an enzyme-linked immunoassay for in vitro measurement of S1P levels in human or animal biological fluids (blood, serum, synovial fluid, cell lysate, and tissue lysate).
This competitive ELISA generates a colorimetric signal inversely proportional to the sample's S1P concentration. Samples are premixed with the Anti-S1P Antibody while the Microtiter Plate is blocked. After blocking, the sample/antibody mixtures are added to the blocked plate for competitive binding. Streptavidin-HRP and colorimetric detection identify Anti-S1P bound to the plate. S1P concentrations are determined using a standard curve. The assay requires 3.5 hours and should be read at 450 nm.

Assay Range: 0.0625 µM to 2 µM

Sensitivity: The lower detection limit of the assay is 0.06 µM[13, 14]

Sample Volume: For duplicate data points 25 µL of each sample is required.

Sample Type: This kit detects S1P in human and animal serum, plasma, tissue homogenate, and cell lysate. The assay is not species specific and has been tested with S1P from human, bovine, equine, caprine, and mouse samples.

Further Validation Studies: Linearity studies were run by serially diluting samples at dilutions 1:15, 1:20, and 1:30 and comparing observed values with expected values. The recoveries observed ranged from 101%-109%[13]. Recovery of spiked S1P standards was tested by adding 3 different S1P concentrations to eight different human plasma samples with various levels of endogenous S1P. The spiked recovery ranged from 90% to 106%.[13]

Assay Performance

For optimal results, follow the provided protocols. Deviating from instructions may compromise kit performance and data accuracy. All samples must be diluted in the Delipidized Serum (K-1904) included in the kit.

Health Hazard Data

The kit includes Delipidized Serum (catalog # K-1904), which should be handled as a potentially biohazardous material. The Delipidized Serum was derived from Human blood donors who were individually tested and shown by FDA approved methods to be negative for antibodies to Human Immunodeficiency Virus (HIV) 1/2 and Hepatitis C Virus (HCV), nonreactive to HIV-1, Hepatitis B surface antigen (HBsAg) and Sexually Transmitted Diseases (RPR). Since no test guarantees the absence of infectious agents, handle it according to universal precautions.

S1P-ELISA Assay Notes

1. Ensure samples are free from debris before adding them to the plate.
2. All samples and standards MUST be diluted in Delipidized Serum (K-1904).
3. Edge effects can cause variability in results between interior and exterior wells of the Microtiter Plate. If concerned or noticing increased variation, avoid using exterior wells. Minimize edge effects by ensuring all reagents are at room temperature before use.
4. We recommend heparin or sodium citrate as the anticoagulant used in plasma samples. EDTA interferes with the S1P-ELISA.
5. Lipid cross reactivity was assessed by testing related lipids (DH-S1P, SPH, DH-SPH, CER, C1P, LPA, PAF, SM, PE, PS, DSPA, LPC, PC) at physiologically relevant levels (10 µM). No cross reactivity was observed except with dihydrosphingosine 1-phosphate (DHS1P) and sphingosylphosphorylcholine (SPC). Until all factors have been tested, the possibility of interference cannot be excluded.
6. When analyzing biological samples, we recommend including a known normal (low S1P) sample and a disease-associated (high S1P) sample alongside your unknown samples. These serve as positive and negative controls and help to differentiate between normal and disease samples.
7. This assay is optimized for detection of S1P in serum and plasma. Sample optimization is highly recommended for other sample types.
8. The S1P-ELISA has been tested with human, bovine, equine and caprine serum sources. The S1P antibody is not species specific. All sample types must be diluted in the Delipidized Serum (K-1904) provided in the kit.
9. Lysis and homogenization buffers can impact the assay. Test internal buffers for interference before running samples. Dilute all samples and standards in Delipidized Serum (K-1904) and lysis/homogenate buffers to minimize buffer effects.

Protocol for the detection of S1P

Read this section and assay notes before starting. This protocol is designed for duplicate reaction points; adjustments are needed for singlet or triplicate points. Begin by placing Streptavidin HRP (K-SEC3h) and Anti-S1P Antibody (K-1901) on ice, while allowing other kit components to reach room temperature before use.

Reagent Preparation

1. PBS Buffer: Add 1 PBS Tablet (K-PTAB) to 200 mL DI water. If using plate washer, mix all 4 PBS tablets (K-PTAB) with 800 mL DI water. Mix until tablet(s) are completely dissolved. Store the prepared PBS Buffer at room temperature.
2. S1P Standard Curve: Prepare a 100 µM S1P Standard by

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adding 50 µL DI water to the lyophilized S1P Standard (K-1905). Vortex to mix and place on ice. The standard curve is designed for serum and plasma samples, but if your samples fall outside this range, a curve with one higher (4 µM) and/or lower (0.0313 µM) standard point can be used. Since cell lysate and tissue homogenate samples tend to have low S1P, the standard curve (step 2.b) has been adjusted accordingly. The hydrated S1P Standard withstands up to three freeze-thaw cycles and should be stored at -20°C when not in use.

a. S1P Standard Curve for the detection of S1P in Serum or Plasma samples Prepare the S1P Standard Curve by adding 12 µL of the 100 µM S1P Standard (K-1905) to 588 µL Delipidized Serum (K-1904) for 2 µM S1P. Then, from the 2 µM S1P make 1:2 serial dilutions using Delipidized Serum to obtain S1P Standards of 1, 0.5, 0.25, 0.125, 0.0625, and 0 µM. These prepared standards can be stored at room temperature for 1-2 hours.

b. S1P Standard Curve for the detection of S1P in Tissue Homogenate or Cell Lysis samples. When using cell lysate or tissue homogenate samples a Standard Curve Diluent needs to be prepared. The diluent must contain the same amount of lysis or homogenate buffer as your prepared samples (step 3b; See Assay Notes 2 and 9). The Standard Curve Diluent is used to dilute the S1P Standards and controls. If a smaller or larger dilution is required for your samples the diluent will need to be adjusted as well.

i. Standard Curve Diluent (1 in 10 sample dilution) Prepare by adding 600 µL of Lysis or Homogenate Buffer to 5,400 µL of Delipidized Serum (K-1904).

ii. S1P Standard Curve Prepare by adding 6 µL of the 100 µM S1P Standard (K-1905) to 594 µL Standard Curve Diluent (step 2.b.i) for 1 µM S1P. From the 1 µM S1P, make1:2 serial dilutions, using the Standard Curve Diluent, to obtain S1P Standards of 0.5, 0.25, 0.125, 0.0625, 0.0313 and 0 µM. These prepared standards can be set at room temperature for 1-2 hours.

3. S1P Sample Preparation

a. Serum and Plasma Samples We recommend a 1:10 dilution of serum and plasma samples in the provided Delipidized Serum (K-1904). For duplicate data points, add 20 µL of sample to 180 µL of Delipidized Serum. This is your working S1P sample.

Important Note: Plasma samples collected in EDTA interfere with the S1P ELISA. We recommend heparin or sodium citrate as anticoagulant.

b. Cell Lysate or Tissue Homogenate For tissue homogenate and cell culture samples we recommend > 30 µg of total protein/well. Cell lysate and tissue homogenate samples tend to have low amounts of S1P. Therefore, it is highly suggested to determine the amount of total protein/well needed for your sample before you run your experiments. Concentration of the sample may be required.

i. Tissue Homogenate and Cell Culture Lysate Samples (30 µg/well): Dilute the tissue homogenate or cell lysis samples to 4 µg/µL total protein with lysis or homogenate buffer.

ii. Further dilute the 4 µg/µL lysis sample 1:10 in Delipidized Serum (K-1904) for a 0.4 µg/µL total protein concentration. For duplicate data points, add 20 µL of sample in 180 µL of Delipidized Serum. This is your working S1P sample

4. Anti-S1P Antibody Preparation Keep the Anti-S1P Antibody (K-1901) on ice. Add 300 µL DI water to rehydrate the lyophilized Anti-S1P Antibody (K-1901). Briefly vortex and place on ice until fully dissolve. Briefly vortex and verify that the pellet is fully dissolved. This stock solution is stable for at least one freeze –defrost cycle and a month's time at -20ºC. To prepare Working Anti-S1P Antibody, add 260 µL of the Anti-S1P Antibody stock to 3.5 mL Diluent Buffer (K-PBST). Mix and place on ice until use. This reagent is not stable at the working concentration and should be prepared immediately before use.

5. Working Streptavidin HRP Dilute the Streptavidin HRP (K-SEC3h) by adding 70 µL Streptavidin HRP (K-SEC3h) to 12 mL Block Solution (K-1903). Mix well. Prepare immediately before use. The Streptavidin HRP (K-SEC3h) can withstand 3 freeze-defrost cycles and should be stored at -20°C. Save the remaining Block Solution for step 2 in the Assay Procedure.

Assay Procedure

1. Remove S1P-coated Microtiter Plate (K-1907) from plastic bag. Block Microtiter Plate by adding 150 µL of Block Solution (K-1903) to each well. Save the remaining Block Solution (K-1903) for dilution of the Streptavidin HRP (K-SEC3h). Place plate seal on Microtiter Plate and incubate at room temperature for 1 hour. Immediately proceed to the next step.
2. Combine your Working S1P Samples or S1P Standards with the Working Anti-S1P Antibody in the Mixing Plate. This step is written for duplicate data points. For single data points, decrease the volumes listed by half. Use the Mixing Plate Layout as a guide.
   a. Add 60 µL of Working Anti-S1P Antibody to each well of the Mixing Plate except the Blank control.
   b. Add 180 µL of each S1P Standard or Working S1P Sample to the Mixing Plate according to your plate layout.
   c. Carefully tap the plate to mix or place on plate shaker at a moderate speed. Once prepared, the Mixing Plate is stable for up to 60 minutes at room temperature. Cover with acetate plate sealer if needed.

   Only half of the wells in the Mixing Plate should be utilized. Each well, once mixed, will be transferred in duplicate to the Microtiter Plate. For this step, nothing should be added to the Blank control wells.
3. After the 1-hour block step (step 1), remove the Block Solution from the Microtiter Plate and wash 3 times with 200 µL/well PBS Buffer. At the end of each wash step, ensure all PBS Buffer is removed from the plate by inverting the plate and blotting it on absorbent paper.
4. Transfer 100 µL of the sample/standard-antibody mixture from the Mixing Plate in duplicate, to each well of the Microtiter Plate. Use the Microtiter Plate Layout as a guide. This transfer is easily accomplished with a multichannel pipette. For best results, prime the pipette 3-6 times before transferring from the Mixing Plate to the Microtiter Plate. Once the transfers are complete place a plate seal on the Microtiter Plate and incubate at room temperature for 1 hour.
5. Wash the Microtiter Plate 3 times with PBS Buffer (step 3).
6. Add 100 µL of Working Streptavidin HRP to each well (including the Blank control wells) of the Microtiter Plate. Place a plate seal on the Microtiter Plate and incubate at room temperature for 1 hour.
7. Wash the Microtiter Plate 3 times with PBS Buffer (step 3).
8. Add 100 µL/well of the TMB Substrate (K-TMB1) and

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incubate for 30 minutes in a dark location. Then add 50 µL 1N Sulfuric Acid (K-STOPt) to each well of the Microtiter Plate to stop the reaction. Read Microtiter Plate absorbance at 450 nm.

Mixing Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	2 µM S1P Standard	-Empty-	Sample 1	-Empty-	Sample 9	-Empty-	Sample 17	-Empty-	Sample 25	-Empty-	Sample 33	-Empty-
B	1 µM S1P Standard	-Empty-	Sample 2	-Empty-	Sample 10	-Empty-	Sample 18	-Empty-	Sample 26	-Empty-	Sample 34	-Empty-
C	0.5 µM S1P Standard	-Empty-	Sample 3	-Empty-	Sample 11	-Empty-	Sample 19	-Empty-	Sample 27	-Empty-	Sample 35	-Empty-
D	0.25 µM S1P Standard	-Empty-	Sample 4	-Empty-	Sample 12	-Empty-	Sample 20	-Empty-	Sample 28	-Empty-	Sample 36	-Empty-
E	0.13 µM S1P Standard	-Empty-	Sample 5	-Empty-	Sample 13	-Empty-	Sample 21	-Empty-	Sample 29	-Empty-	Sample 37	-Empty-
F	0.06 µM S1P Standard	-Empty-	Sample 6	-Empty-	Sample 14	-Empty-	Sample 22	-Empty-	Sample 30	-Empty-	Sample 38	-Empty-
G	0 µM S1P Standard	-Empty-	Sample 7	-Empty-	Sample 15	-Empty-	Sample 23	-Empty-	Sample 31	-Empty-	Sample 39	-Empty-
H	Blank	-Empty-	Sample 8	-Empty-	Sample 16	-Empty-	Sample 24	-Empty-	Sample 32	-Empty-	Sample 40	-Empty-

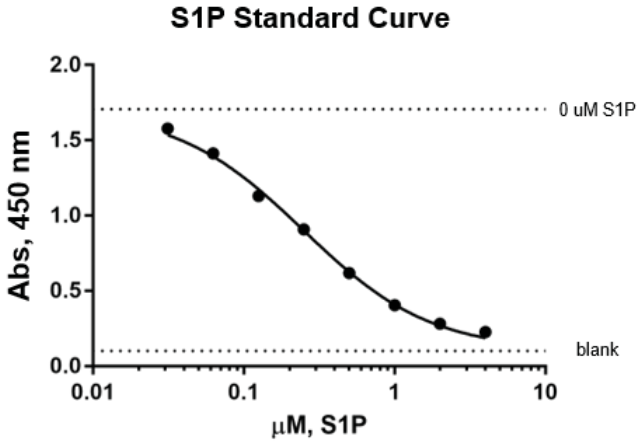
Microtiter Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	2 µM S1P Standard	2 µM S1P Standard	Sample 1	Sample 1	Sample 9	Sample 9	Sample 17	Sample 17	Sample 25	Sample 25	Sample 33	Sample 33
B	1 µM S1P Standard	1 µM S1P Standard	Sample 2	Sample 2	Sample 10	Sample 10	Sample 18	Sample 18	Sample 26	Sample 26	Sample 34	Sample 34
C	0.5 µM S1P Standard	0.5 µM S1P Standard	Sample 3	Sample 3	Sample 11	Sample 11	Sample 19	Sample 19	Sample 27	Sample 27	Sample 35	Sample 35
D	0.25 µM S1P Standard	0.25 µM S1P Standard	Sample 4	Sample 4	Sample 12	Sample 12	Sample 20	Sample 20	Sample 28	Sample 28	Sample 36	Sample 36
E	0.13 µM S1P Standard	0.13 µM S1P Standard	Sample 5	Sample 5	Sample 13	Sample 13	Sample 21	Sample 21	Sample 29	Sample 29	Sample 37	Sample 37
F	0.06 µM S1P Standard	0.06 µM S1P Standard	Sample 6	Sample 6	Sample 14	Sample 14	Sample 22	Sample 22	Sample 30	Sample 30	Sample 38	Sample 38
G	0 µM S1P Standard	0 µM S1P Standard	Sample 7	Sample 7	Sample 15	Sample 15	Sample 23	Sample 23	Sample 31	Sample 31	Sample 39	Sample 39
H	Blank	Blank	Sample 8	Sample 8	Sample 16	Sample 16	Sample 24	Sample 24	Sample 32	Sample 32	Sample 40	Sample 40

Quantification of Samples

To determine relative sample values, generate a best-fit curve using the S1P standards. The 8-point S1P standard curve (shown below) was constructed using nonlinear regression analysis via GraphPad Software, employing a semi-log [sigmoidal dose-response (variable slope)] model. For optimal results, constrain the top and bottom of the curve using the 0 µM S1P and blank controls. Ensure that all sample S1P concentrations account for the dilution factor from step 3 of reagent preparation.

Table 1: S1P Standards	
µM S1P	pmol/well S1P
4.0	300
2.0	150
1.0	75
0.5	37.5
0.25	18.75
0.125	9.75
0.0625	4.5
0.03125	2.25



When analyzing tissue homogenates or cell lysates, plot the S1P standard curve in units of pmol/well (Table 1). Interpolated values should then be normalized to either the total protein content (g) or tissue mass (mg), as appropriate.

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Preparation of Plasma

Materials and Equipment

- Human blood sample.
- Vacutainer tubes containing anticoagulant
- Serological pipettes of appropriate volumes (sterile), centrifuge tubes, cryovials
- Benchtop centrifuge (NOT refrigerated) with swing-out rotor and appropriate carriers

Procedure

1. Draw blood into vacutainer tube(s) containing anticoagulant following local standard operating procedures (this may vary depending on manufacturer). Be sure to draw the full volume to ensure the correct blood to anticoagulant ratio.
2. Invert vacutainer tubes carefully 10 times to mix blood and anticoagulant and store at room temperature until centrifugation.
3. Samples should undergo centrifugation immediately. This should be carried out for a minimum of 10 minutes at 1000-2000 RCF (generally 1300 RCF) at room temperature (refer to speeds and times recommended by manufacturer). Do not use the brake to stop the centrifuge.
4. This will give three layers: (from top to bottom) plasma, leucocytes (buffy coat), erythrocytes. Carefully aspirate the supernatant (plasma) at room temperature and transfer to a centrifuge tube. Do not disrupt the cell layer or transfer any cells.
5. Inspect plasma for turbidity. Turbid samples should be centrifuged and aspirated again to remove any remaining insoluble material.
6. Aliquot plasma into cryovials and store at –80°C. Ensure that the cryovials are adequately labeled with the relevant information, including details of additives present in the blood.

Important Note: Plasma samples collected in EDTA interfere with the S1P ELISA. We recommend heparin or sodium citrate as anticoagulant.

Preparation of Serum

Materials and Equipment

- Human blood sample
- Vacutainer tubes (containing either no additive or a clot activator)
- Serological pipettes of appropriate volumes (sterile), centrifuge tubes, cryovials
- Benchtop centrifuge (NOT refrigerated) with swing-out rotor and appropriate carriers

Procedure

1. Draw whole blood into vacutainer tube(s) containing no anticoagulant following local standard operating procedures. Draw approximately 2½ times the volume needed for use e.g. 10 mL blood for 4 mL serum.
2. Incubate in an upright position at room temperature for 30-45 min (no longer than 60 min) to allow clotting. If you use a clot-activator tube, invert carefully 5-6 times to mix the clot activator and blood before incubation.
3. Centrifuge for 15 minutes at manufacturer's recommended speed (usually 1,000-2,000 RCF). Do not use the brake to stop the centrifuge.
4. Carefully aspirate the supernatant (serum) at room temperature and transfer into a centrifuge tube, taking care not to disturb the cell layer or transfer any cells. Use a clean pipette for each tube. Inspect the serum for turbidity. Turbid samples should be centrifuged and aspirated again to remove remaining insoluble matter.
5. Aliquot into cryovials and store at –80 °C.

Preparation of Tissue Homogenate

The following protocol was utilized for the determination of S1P concentrations in embryonic and adult kidneys. It was provided by an outside collaborator and has not been validated internally.

Procedure

1. Kidneys were homogenized and sonicated in the following homogenization buffer: 20 mM Tris-HCl, pH 7.4; 20% glycerol; 1 mM β-mercaptoethanol; 1 mM EDTA; 1 mM Naorthovanadate; 15 mM NaF; 1 mM PMSF; protease inhibitor cocktail (Sigma); 0.5 mM deoxypyridoxine; 40 mM β-glycerophosphate.
2. Total protein concentration was measured.
3. The homogenates were then frozen at -80ºC until analysis.

Preparation of Cultured Cell Lysate

The S1P ELISA was tested with cell lysates from PC-3, DU-145, and LNCaP cell lines from ATCC. The cell lysate protocol was provided by Dr. Shahriar Koochekpour, School of Medicine, LSU Health Sciences Center, New Orleans, LA.

Analysis Notes

- No cross reactivity with base cell culture media (DME high Glucose, RPMI 1640, SF900 II SFM, BacVector Insect Media, Dulbecco's PBS, HBSS)
- Lipid extraction and sonication do not work – no detectable S1P.
- Media contains FBS. Use of serum free media is recommended.

Table 2: Lysis Buffer Preparation Table.
Prepare and store at 4°C

Reagent	Stock Concentration	For 100 mL	For 500 mL
PIPES	20 mM	604.8 mg	3.024 g
NaCl	150 mM	876.6 mg	4.383 g
EGTA	1 mM	38.04 mg	190.2 mg
Triton X-100	1% V/V	1 mL	5 mL
$MgCl_2$	1.5 mM	30.5 mg	152.5 mg

Add immediately before use

Reagent	Stock Concentration	For 5 mL
SDS	10%	50 µL
Protease inhibitor Cocktail	25X	200 µL
Na-Orthovanidate	1 M	5 µL

Procedure

1. All reagents should be freshly prepared, and all steps should be performed on ice.
2. 75 cm flasks were cultured up to 80-85% confluence, either serum starved for 24 hour or wash with PBS X 2.
3. Cells were lysed in 400 µL of Lysis Buffer with 20 mM PIPES, 150 mM NaCl, 1 mM EGTA, 1% V/V Triton X-100, 1.5 mM MgCl2, 0.1% SDS, 1 mM NaOrthovanidate, 1X Protease

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inhibitor cocktail (without EDTA), pH 7. See Lysis buffer preparation in table 2 below.
4. The clarified lysate was frozen immediately at -80ºC. Protein concentration was measured by the BCA method.
5. Diluted cell lysate samples (1:10 in Delipidized Serum, K-1904) are then analyzed with the S1P-ELISA Assay.

Lysis buffer preparation

Mix solution with stir bar for 20 minutes and adjust pH to 7.4 using NaOH or HCl until a clear solution is obtained.
Filter the solution using a 0.45µm filter. This is stable for 6-12 months and must be stored on ice or 4ºC.
To make the final "lysis buffer" add 50 µL of 10% SDS, 200 µL of 25X protease inhibitor cocktail and 5 µL 1 M NaOrthovanadate for each 5 mL of working lysis buffer. Vortex mixture and place on ice.

References

General References

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Anti-S1P Antibody (LT1002) Reference

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Selected S1P-ELISA (K-1900) References

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S1P in Kidney Homogenates (Results validated by liquid chromatography-tandem mass spectrometry)

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Related Products

Products	Catalog Number
Assays and Enzymes	
S1P Activity Assay	K-3500
S1P Inhibitor Screen	K-4400
Sphingosine Kinase 1 and 2	E-K068, E-K069
Antibodies	
Anti-S1P	Z-P300
Lipids and Beads	
S1P	S-2000
Biotin-S1P	S-200B
S1P coated Beads	S-6110
Agonists	
FYT720 FYT720-Phosphate	B-0720 B-0721

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