Sphingosine 1-Phosphate ELISA Kit

Enzyme Immunoassay for the quantification of Sphingosine 1-Phosphate (S1P) in serum, plasma, tissue homogenate and cell culture lysate samples.

Catalog number: ARG81133

Package: 96 wells

TABLE OF CONTAINS

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INTRODUCTION

Sphingosine-1-phosphate (S1P) is a signaling sphingolipid, also known as lysosphingolipid. S1P is a bioactive lipid second messenger that regulates diverse biological processes in health and disease. Although S1P is of importance in the entire human body, it is a major regulator of vascular and immune systems. In addition, it might be relevant in the skin. In the vascular system, S1P regulates angiogenesis, vascular stability, and permeability. In the immune system, it is now recognized as a major regulator of trafficking of T- and B-cells. S1P interaction with its receptor S1PR1 is needed for the egress of immune cells from the lymphoid organs (such as thymus and lymph nodes) into the lymphatic vessels. Inhibition of S1P receptors was shown to be critical for immunomodulation. S1P has also been shown to directly suppress TLR mediated immune response from T cells. The levels of S1P (in a range of 5–40 µmol/L) are 5 to 10 times up-regulated in ovarian cancer patients' ascites. S1P at this physiological concentration stimulates migration and invasion of epithelial ovarian cancer cells but inhibits migration of normal ovarian surface epithelial cells. Most (more than 90%) ovarian cancers arise from the epithelium of the ovary. Therefore, extracellular S1P could have an important role in cancer progression by promoting migration of epithelial ovarian cancer cells.

Ozonization of human blood is associated with increased concentrations of S1P in the plasma.

In addition, S1P modulates the proliferation of skin cells. This in particular applies to keratinocytes while fibroblasts are not addressed in this way, apart from cell growth and differentiation While S1P suppresses epidermal

proliferation as the glucocorticoids do, it differs from them in so far, as proliferation of dermal fibroblasts is not reduced. In fact, S1P even activates fibroblast-derived extracellular matrix protein production.

[Provide by Wikipedia: Sphingosine-1-phosphate]

PRINCIPLE OF THE ASSAY

This is a competitive enzyme Immunoassay for the quantification of Sphingosine 1-Phosphate (S1P) in serum, plasma, tissue homogenate and cell culture lysate samples.

This assay employs the competitive quantitative enzyme immunoassay technique. The antigen has been pre-coated onto a microtiter plate. Standards or samples are pre-mixed with biotin-conjugated anti-S1P antibody and the S1P present in samples or standard will bind to the anti-A1P antibody. After mixing, the sample/anti-S1P antibody or standard/anti-S1P antibody mixture are adding into the blocked antigen-coated microtiter plate. The S1P on the plate will compete the antibody binding site with S1P in samples or standards. Sample S1P/anti-S1P antibody or standard S1P/anti-S1P antibody complex are removed by washing. Then an HRP-conjugated streptavidin is added to each well and incubated. After washing away any unbound antibody conjugate, a substrate solution (TMB) is added to the wells and color develops in inverse-proportion to the amount of S1P present in the samples. The color development is stopped by the addition of STOP solution and the intensity of the color is measured at a wavelength of 450nm ±2nm. The concentration of S1P in the sample is then determined by comparing the O.D of samples to the standard curve.

MATERIALS PROVIDED & STORAGE INFORMATION

Upon receipt must store TMB Substrate at 4°C and all other components at –20°C. All components and solutions should be protected from excessive light and heat. Use the kit before expiration date.

Component	Quantity	Storage information
S1P coated Microtiter Strips	12 strips X 8 wells	4°C or below
Mixing Plate (U-bottom plate)	1 plate	4°C or below
Blocking reagent	1X 30 ml (Ready-to-use)	-20°C
Assay Buffer	1X 15 ml (Ready-to-use)	-20°C
S1P Standard	1 vial (Lyophilized)	-20°C
Biotin conjugated Anti-S1P antibody	1 vial (Lyophilized)	-20°C
Antibody Diluent	1X 5 ml (Ready-to-use)	-20°C
PBS tablets	4 Tablets	-20°C
HRP-conjugated streptavidin	1X 80 µl	-20°C
TMB substrate	1 X 12 ml (Ready-to-use)	4°C (Protect from light)
STOP solution	1 X 8 ml (Ready-to-use)	4°C
Adhesive foil	2 pieces	RT

MATERIALS REQUIRED BUT NOT PROVIDED

- Microplate reader capable of measuring absorbance at 450nm
- Multichannel pipettes, Pipettes and pipette tips
- Deionized or distilled water
- Plate Shaker (optional)
- Automated microplate washer (optional)

TECHNICAL HINTS AND PRECAUTIONS

- Wear protective gloves, clothing, eye, and face protection especially while handling blood or body fluid samples.
- Upon receipt, this 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 use.
- All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
- All kit reagents (except conjugated antibody and streptavidin) and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
- Conjugated antibody and streptavidin should be placed on ice before use.
- Make sure the TMB substrate is defrosted, gently mixed and equilibrated to room temperature before use.
- Ensure samples are free from debris before adding to the plate.
- When analyzing biologic samples we recommended to run a known normal sample (low S1P) and a disease sample (high S1P) in conjunction with your unknown samples. These will serve as positive and negative controls to aid in distinguishing between normal healthy samples and disease samples.
- This assay is optimized for detection of S1P in serum and plasma. Sample optimization is highly recommended for other sample types.

- Once the test has been started, all steps should be completed without interruption. Make sure that the required reagents, materials and devices are prepared ready at the appropriate time.
- Ensure complete reconstitution and dilution of reagents prior to use.
- It is highly recommended that the standards and samples be assayed in duplicates.
- Change pipette tips between the addition of different reagent or samples.

SAMPLE COLLECTION & STORAGE INFORMATION

The sample collection and storage conditions listed below are intended as general guidelines, user may verified for the optimize protocol. Sample stability has not been evaluated.

Plasma - Collect plasma using heparin or sodium citrate an anticoagulant. Plasma collected by EDTA is not recommend, since EDTA interferes the assay. Do not use haemolytic or lipemic samples. Centrifuge for 10 minutes at 1000-2000 x g (generally 1300 x g) immediately of collection. Do not use brake to stop centrifuge. Carefully aspirate the supernatant (plasma) at room temperature and transfer to a centrifuge tube. Take care not to disrupt the cell layer or transfer any cells (do not collect buffy coat either). Inspect plasma for turbidity. Turbid samples should be centrifuged and aspirated again to remove remaining insoluble matter. Collect the supernatant. Assay immediately or aliquot and store samples at ≤ -80 °C. Avoid repeated freeze-thaw cycles. Avoid exposure to direct sunlight.

Serum- Use a serum separator tube (SST) and allow samples to clot for 30 minutes before centrifugation for 15 minutes at 1000-2000 x g. Do not use brake to stop centrifuge. Inspect plasma for turbidity. Turbid samples should be centrifuged and aspirated again to remove remaining insoluble matter. Collect the supernatant. Collect serum and assay immediately or aliquot and store samples at ≤ -80 °C. Avoid repeated freeze-thaw cycles.

Tissue Homogenate

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

Tissue Homogenate Procedure:

1. Kidneys were homogenized and sonicated in the following homogenization buffer: 20 mM Tris-HCl, pH 7.4; 20% glycerol; 1mM 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. Centrifuge for 15-30 minutes at 14000 x rpm at 4°C, collect the supernatant.
3. Total protein concentration was measured.
4. Aliquot and store the tissue lysate samples at ≤ -80°C.

Cultured Cell Lysate

The S1P ELISA kit was tested with cell lysates from PC-3, DU-145, and LNCaP cell lines from ATCC. It is recommended use of serum free media for the culturing before collection. All reagents should be freshly prepared and all steps should be performed on ice.

1. 75 cm flasks were cultured up to 80-85% confluence, either serum starved for 24 hour or wash with PBS X 2.
2. 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 $MgCl_2$, 0.1% SDS, 1 mM Na-Orthovanidate, 1X Protease inhibitor cocktail (without EDTA), pH 7.
3. Centrifuge for 15-30 minutes at 14000 x rpm at 4°C, collect the supernatant.
4. Protein concentration was measured by the BCA method.
5. Aliquot and store the clarified lysate samples at ≤ -80 °C.
6. Diluted cell lysate samples (1:10 in Assay Buffer) are then analyzed with the S1P-ELISA Assay.

Notes:

a. The kit is no cross reactivity with base cell culture media (DME high Glucose, RPMI 1640, SF900 II SFM, BacVector Insect Media, Dulbecco's PBS, HBSS)
b. Lipid extraction and sonication do not work – no detectable S1P.
c. Lysis and homogenization buffers may affect the assay. Always test internal lysis and homogenization buffers in the assay for interference before running samples. All samples and standards must be diluted in Assay Buffer and lysis/homogenate buffers to reduce potential buffer effects.
d. Media contains FBS. Use of serum free media is recommended.

REAGENT PREPARATION

- PBS: Reconstitute the one tablet of PBS with 200 ml of distilled water and mix well until the tablet is completely dissolved. The reconstituted PBS can be store at room temperature.
- Working Anti-S1P Antibody:

  - Reconstitution: Keep the lyophilized Anti-S1P Antibody on ice. Reconstitute the Anti-S1P Antibody with 300 µl of distilled water, briefly vortex and keep the vial on ice for 15 min to completely dissolve. Aliquot and store this stock antibody solution at -20°C for up to one month and the stock antibody solution is stable for at least one freeze–thaw cycle. Briefly vortex and verify if it is fully dissolved before working Anti-S1P Antibody preparation.

  - Working anti-S1P antibody preparation: This reagent is not stable at the working concentration and should be prepared immediately before use. Dilute Anti-S1P Antibody stock with Antibody Diluent Buffer. 60 µl of Working anti-S1P antibody is needed for each well. For 96 wells to be used. Add 260 µl of the Anti-S1P Antibody stock to 3.5 mL of Antibody Diluent Buffer. Mix well and keep on ice until use.
- Working HRP-conjugated Streptavidin solution: The HRP-Streptavidin stock should be stored at-20°C and it is stable for 3 freeze-thaw cycles. Prepare the working HRP-conjugated Streptavidin solution immediately before use. 100µl is needed per-well, prepare the needed volume only. Dilute the HRP-conjugated Streptavidin stock by adding 70 µl of HRP-Streptavidin stock to 12 ml Blocking reagent, mix thoroughly. Mix well and keep on ice until use.

- **Standard**:

  Reconstitute the standard with **50 µL** of **distilled water** to yield a stock concentration of **100 µM.** Vortex to mix and place on ice. Make sure the standard is dissolved completely before making serial dilutions. The stock standard solution should be aliquoted & stored at-20°C. Avoid repeated freeze-thaw cycles.

  - For serum and plasma samples:

  Add **12 µL** of the reconstituted 100 µM S1P Standard to **588 µL** of Assay Buffer yield a high concentration standard of **2 µM** S1P.

  The Assay Buffer serves as zero standard (0 ng/ml), and the rest of the standard serial dilution can be diluted as according to the suggested concentration below: **2 µM, 1 µM, 0.5 µM, 0.25 µM, 0.125 µM, 0.0625 µM and 0 µM**.

Dilution table for S1P standard preparation for serum and plasma samples:

S1P Concentration (µM)	Volume of Assay Buffer (µl)	Volume of standard (µl)
2	588	12 (100 µM stock)
1	300	300 (2 µM)
0.5	300	300 (1 µM)
0.25	300	300 (0.5 µM)
0.125	300	300 (0.25 µM)
0.0625	300	300 (0.125 µM)
0	300	0

- For Tissue Homogenate or Cultured Cell Lysate samples

Lysate Sample Standard Diluent Buffer: When using Tissue Homogenate or Cultured Cell Lysate samples, the lysate buffer as your prepared samples should be included in the standard diluent. Add lysate buffer 1:10 in Assay buffer to get a *Lysate Sample Standard Diluent Buffer*. E.g. Add 600 µl of lysate buffer into 5400 µl of Assay buffer.

Dilution: Since lysate samples might contain S1P lower then serum or plasma samples. We recommended the suggested concentration as below for Tissue Homogenate or Cultured Cell Lysate samples.

Add 6 µL of the reconstituted 100 µM S1P Standard to 594 µL *Lysate Sample Standard Diluent Buffer* (refer to above) yield a high concentration standard of 1 µM S1P.

The *Lysate Sample Standard Diluent Buffer* serves as zero standard (0 ng/ml), and the rest of the standard serial dilution can be diluted as according to the suggested concentration below: **1 µM, 0.5 µM, 0.25 µM, 0.125 µM, 0.0625 µM, 0.03125 µM and 0 µM.**

Dilution table for S1P standard preparation for tissue homogenate or cultured cell lysate samples:

S1p Concentration (µM)	Volume of *Standard Diluent Buffer* (µl)	Volume of standard (µl)
1	594	6 (100 µM stock)
0.5	300	300 (1 µM)
0.25	300	300 (0.5 µM)
0.125	300	300 (0.25 µM)
0.0625	300	300 (0.125 µM)
0.03125	300	300 (0.0625 µM)
0	300	0

Note: If the samples are expected to run outside of the standard range, an S1P Standard concentration with one standard point higher (4 µM) and/or lower (0.0313 µM) can be used. The S1P Standard can stable up to 3 freeze-thaw cycles and should be stored at -20°C when not in use.

- Samples

  Serum and Plasma Samples: We recommend dilute the **serum and plasma** samples in Assay Buffer at 1:10 dilution before assay. E.g. For duplicate samples, add 20 µl of sample to 180 µl of Assay Buffer to make sure have minimal 180 µl of each sample for the further assay to incubate with anti-S1P antibody.

  Tissue Homogenate or Cultured Cell Lysate samples: We recommend add at least 30 µg of total protein per well. Tissue Homogenate or Cultured Cell Lysate samples might contains low amounts of S1P. Therefore, it is highly suggested to determine the amount of total protein per well needed for your sample before assay.

  - To get 30 µg of total protein per well, dilute the tissue homogenate or cell lysate samples to 4 µg/µl of total protein with cell lysis buffer or tissue homogenate buffer as your prepared samples.

  - **Further dilute** the 4 µg/µl lysis sample 1:10 with **Assay Buffer** to get a 0.4 µg/µl concentration of total protein. E.g. For duplicate samples, add 20 µl of sample to 180 µl of Assay Buffer to make sure have minimal 180 µl of each sample for the further assay to incubate with anti-S1P antibody.

ASSAY PROCEDURE

All materials (except working anti-S1P antibody and HRP-streptavidin should keep on ice before use) should be equilibrated to room temperature (RT) 15 min before use. Samples and standards should be assayed in duplicates.

1. Remove excess S1P coated microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal it.
2. Plate blocking: Add **150 µl** of **Blocking reagent** to each well of S1P coated Microtiter Strips. Cover the plate with foil and incubate for **1 hour at room temperature.**
3. Biotin-conjugated antibody mixture:
   a、 While block the non-specific binding on the wells of S1P coated microplate strips with blocking buffer. (The below preparation steps are for duplicates samples, if single sample is used, please decrease the volumes at below step by half.) Add **60 µL** of **Working Anti-S1P Antibody** to each well of the **U-bottom plate** except the Blank control.
   b、 Add **180 µL** of each **S1P Standard** or **Working S1P Sample** into the appropriate wells of the **U-bottom plate**. (For mixing duplicates samples, the mixture could only be done in row 1, 3, 5, 7, 9, 11, and keep row 2, 4, 6, 8, 10, 12 empty to avoid contamination.)
   c、 Carefully tap the mixing Plate or place the mixing Plate on a shaker at a moderate speed to mix thoroughly. Cover with foil if needed. The mixture will be transferred in duplicate to the S1P coated Microtiter Plate.
   d、 The mixture in Mixing Plate is stable for **up to 60 minutes** at room temperature.

e、 At this step, nothing should be added to the Blank control wells.

f、 The volume at above is design for duplicate samples. For single sample, decrease the volumes listed by half.

4. After the 1 hour blocking step (step 2), remove the Blocking reagent from the Microtiter plate.
5. Aspirate each well and wash, repeating the process 2 times for a **total 3 washes**. Wash by filling each well with **PBS (200 µl)** using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each is essential to good performance. After the last wash, remove any remaining PBS by aspirating, decanting or blotting it on clean absorbent paper towels.
6. Transfer **100 µl** of the **sample/standard-antibody mixture** from the **U-bottom plate** (**step 3**), in duplicate, to each well of the S1P coated Microtiter Plate. (Carefully tap the **U-bottom plate** or prime the pipette 3-6 times with multi-channel pipette to mix the mixture thoroughly before transfer.) Keep 2 wells empty as blank control.
7. Cover the plate with foil and incubate for **1 hour at room temperature**.
8. **Wash** the plate as according to **step 5.**
9. Add **100 µl** of **Working HRP-conjugated Streptavidin** solution into all wells (including the Blank control wells) of the Microtiter Plate.
10. Cover the plate with foil and incubate for **1 hour at room temperature**.
11. **Wash** the plate as according to **step 5**.
12. Add **100 µl** of **TMB substrate solution** into each well. Incubate for **30 mins at room temperature**. Avoid exposure to light.
13. Add **50 µl** of **Stop Solution** to each well and shake lightly to ensure homogeneous mixing.

14. Read the OD with a microplate reader at 450nm immediately.

CALCULATION OF RESULTS

1. Calculate the average absorbance values for each set of standards, controls and samples.

2. For best results, constrain the standard curve top & bottom using the 0 µM S1P & Blank controls.

3. Using log-log, semi-log or linear graph paper, construct a standard curve by plotting the mean absorbance obtained from each standard against its concentration with absorbance value on the vertical (Y) axis and concentration on the horizontal (X) axis.

4. Using the mean absorbance value for each sample determine the corresponding concentration from the standard curve.

5. Automated method: The results in the IFU have been calculated automatically using a 4 PL (4 Parameter Logistics) curve fit. 4 Parameter Logistics is the preferred method. Other data reduction functions may give slightly different results.

6. arigo provides GainData®, an in-house development ELISA data calculator, for ELISA data result analysis. Please refer our GainData® website for details. (https://www.arigobio.com/elisa-analysis)

7. Since the samples have been diluted by 1:10, the concentration read from the standard curve must be further converted by the appropriate dilution factor according to the sample preparation procedure as described above.

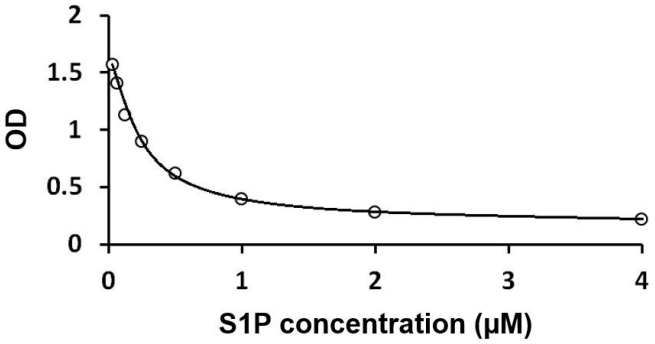
8. For tissue homogenate or cell lysate samples the S1P standard curve should

be graphed in pmol/well S1P (see table as below). The interpolated sample values should then be normalized with grams of total protein or mgs of tissue.

	Concentration of standards							
S1P (µM)	4	2	1	0.5	0.25	0.125	0.0625	0.03125
S1P (pmol/well)	300	150	75	37.5	18.75	9.75	4.5	2.25
Conversion	S1P (µM) x 75 = S1P (pmol/well)							

EXAMPLE OF TYPICAL STANDARD CURVE

The following data is for demonstration only and cannot be used in place of data generations at the time of assay.



QUALITY ASSURANCE

Sensitivity

0.03125 µM

Assay Range

4 µM - 0.03125 µM (dependent on the sample type)

Specificity

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 DH-S1P (dihydrosphingosine 1-phosphate) and SPC (sphingosylphosphorylcholine). Until all factors have been tested, the possibility of interference cannot be excluded.