Version 18-08/24

User's Manual

Sphingosine 1-Phosphate ELISA Kit

REF **DEIA-XYZ5**

Σ **96T**

RUO

This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The S1P-ELISA is an enzyme-linked immunoassay designed for the in vitro measurement of S1P levels in human or animal biological fluids (blood, serum, synovial fluid, cell lysate, and tissue lysate).

General Description

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 proliferative1, pro-angiogenic2, and anti-apoptotic3 sequence of events that contributes to the progression of cancer, diabetes, and osteoporosis. SphK1 has been shown to be up-regulated 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 detection4,5. 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 immune6 and vascular regulation7. S1P is also known for its direct role as a second messenger during inflammation7 and the SPhK/S1P/S1P-receptor signaling family 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.

Principles of Testing

The S1P-ELISA is a competitive ELISA in which the colorimetric signal is inversely proportional to the amount of S1P present in the sample. The samples are pre-mixed with the Anti-S1P Antibody while the Microtiter Plate is blocked. Once the block step is complete, the sample/Anti-S1P Antibody mixtures are added to the Blocked Microtiter Plate for competitive binding. Streptavidin- HRP and colorimetric detection is used to detect the Anti-S1P bound to the Microtiter Plate. The concentration of S1P in the sample is determined using a standard curve of known amounts of S1P. This assay should be read at 450 nm and requires 3.5 hours to run.

Reagents And Materials Provided

1. Anti-S1P Antibody: Lyophilized Powder
2. Block Solution: 30 mL
3. Delipidized Serum (DHS): 15 mL
4. Sphingosine 1-Phosphate (S1P) Standard: 50 µL
5. S1P Coated Microtiter Plate (12 X 8 strip-well plate): 1 Plate
6. PBS Tablets: 4 Tablets
7. Diluent Buffer: 5 mL

8. Streptavidin HRP: 80 µL
9. TMB Substrate: 12 mL
10. 1N Sulfuric Acid: 10 mL
11. Yellow U-bottom Mixing Plate: 1 Plate
12. Plate Seals Clear Acetate Sheet, 1 side adhesive: 2 Seals

Materials Required But Not Supplied

- 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)

Storage

Upon receipt, Part 1 of the S1P-ELISA kit must be stored at -20°C and Part 2 (TMB Substrate) must be stored at 4°C. Under proper storage conditions, the kit components are stable for at least 6 months from date of receipt. Opened and reconstituted solutions are less stable. All components and solutions should be protected from excessive light and heat.

Specimen Collection And Preparation

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 brake to stop 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. Take care not to disrupt the cell layer or transfer any cells.

5. Inspect plasma for turbidity. Turbid samples should be centrifuged and aspirated again to remove remaining insoluble matter.
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 the 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 using a clot- activator tube, invert carefully 5-6 times to mix 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 brake to stop 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. Ensure that the cryovials are adequately labeled with the relevant information, including details of additives present in the blood.

Preparation of 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; 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 -80°C 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.

Cell Lysis Procedure:

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 X2.
2. Cells were lysed in 400 µL of Lysis Buffer with 20 mM PIPES, 150 mM NaCl, 1mM EGTA, 1% V/V Triton X-100, 1.5 mM MgCl2, 0.1% SDS, 1 mM NaOrthovanidate, 1X Protease inhibitor cocktail (without EDTA), pH 7. See Lysis buffer preparation in table below.
3. The clarified lysate was frozen immediately at -80°C.
4. Protein concentration was measured by the BCA method.
5. Diluted cell lysate samples (1:10 in Delipidized Serum) are the nanalyzed with the S1P-ELISA Assay.

Lysis Buffer: Prepare and store 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	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	
NaOrthovanidate	1 M	5 µL	

Lysis buffer preparation:

1. Mix solution with stirbar for 20minutes and adjust pH to 7.4 using NaOH or HCl until a clear solution is obtained.
2. Filter the solution using a 0.45 µm filter .This is stable for 6-12 months and must be stored on ice or 4°C.
3. 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.

Reagent Preparation

1. PBS Buffer: Add 1 PBS Tablet to 200 mL DI water. If using plate washer, mix all 4 PBS Tablets with 800 mL DI water. Mix until tablet(s) are completely dissolved. Store prepared PBS Buffer at room temperature.
2. S1P Standard Curve: The S1P standard curve has been designed for serum and plasma samples. If your samples are expected to run outside of this curve, an S1P Standard curve with one standard point higher (4 µM) and/or lower (0.0313 µM) in concentration can be used. Cell lysate and tissue homogenate samples tend to have low S1P; therefore the standard curve (step 2.b) has been adjusted accordingly. The S1P Standard can handle up to 3 freeze-defrost 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 to 588 µL Delipidized Serum 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 Standard Curve Diluent must contain the same amount of lysis or homogenate buffer as your prepared samples (step 3b - See also 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 Standard Curve Diluent will need to be adjusted as well.

i. Standard Curve Diluent: Prepare by adding 600µL of Lysisor Homogenate Buffer to 5,400µL of Delipidized Serum.

ii. S1P Standard Curve: Prepare by adding 6µL of the 100 µM S1P Standard to 594µL 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. 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 the anticoagulant.

b. Cell Lysate or Tissue Homogenate: For tissue homogenate and cell culture samples we recommend> 30 µ g 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.

- Further dilute the 4 µg/µL lysis sample 1:10 in Delipidized Serum 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 on ice. Add 150 µL DI water to rehydrate the lyophilized Anti-S1P Antibody. Briefly vortex and place on ice for 15 min to fully dissolve. Briefly vortex and verify that it is fully dissolved. This stock solution is stable for at least one freeze -defrost cycle and a

month's time at -20°C. To prepare Working Anti-S1P Antibody, add 130 µL of the Anti-S1P Antibody stock to 3.5 mL Diluent Buffer. Mix and place on ice until use. This reagent is not stable at the working concentration and should be prepared immediately before use. This reagent is not sold separately from the kit.

5. Working Streptavidin HRP: Dilute the Streptavidin HRP by adding 18 µL Streptavidin HRP to 13 mL Block Solution. Mix well. Prepare immediately before use. The Streptavidin HRP 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

Assay Notes

1. Ensure samples are free from debris before adding to the plate.
2. All samples and standards MUST be diluted in Delipidized Serum.
3. Be cautious of edge effects. The results from wells at the edge of the Microtiter Plate may vary from the interior wells of the

plate. If concerned about the edge effect or have observed increased variation with the exterior wells then do not use the exterior wells of the Microtiter Plate. To reduce edge effects ensure 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, LP A, P AF, 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 biologic samples we advise running a known normal (low) S1P sample and a disease (high) S1P sample 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.
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 provided in the kit.
9. 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 Delipidized Serum and lysis/homogenate buffers to reduce potential buffer effects.

Please read this entire section and assay notes before beginning assay. This protocol has been developed for duplicate reaction points. If singlet or triplicate points are required, the protocol will need to be adjusted accordingly. To begin, place the Streptavidin HRP and Anti-S1P Antibody on ice. Allow the remaining kit components to warm to room temperature before use.

1. Remove S1P-coated Microtiter Plate from plastic bag. Block Microtiter Plate by adding 150 µL of Block Solution to each well. Save the remaining Block Solution for dilution of the Streptavidin HRP. 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 Yellow Mixing Plate. This step is written for duplicate data points. For single data points, decrease the volumes

listed by half. Use the Yellow Mixing Plate Layout as a guide.

a. Add 60 µL of Working Anti-S1P Antibody to each well of the Yellow Mixing Plate except the Blank control.

b. Add 180 µL of each S1P Standard or Working S1P Sample to the Yellow 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 Yellow 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 Yellow 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.

Yellow 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-

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 Yellow 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 multi-channel pipette. For best results, prime the pipette 3-6 times before transferring from the Yellow 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.

Microtiter Plate Layout:

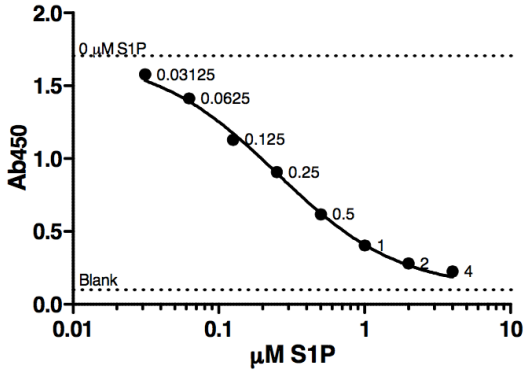
	1	2	3	4	5	6	7	8	9	10	11	12
A	2 µM S1P Standard	2 µM S1P Standard	Sample # 1	Sample # 1	Sample # 9	Sample # 9	Sample # 17	Sample # 17	Sample # 25	Sample # 25	Sample # 33	Sample # 33
B	1 µM S1P Standard	1 µM S1P Standard	Sample # 2	Sample # 2	Sample # 10	Sample # 10	Sample # 18	Sample # 18	Sample # 26	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

5. Wash the Microtiter Plate 3 times with PBS Buffer (see 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 (see step 3).
8. Add 100 µL/well of the TMB Substrateand 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.

Typical Standard Curve

Generate a best fit curve for the S1P standards in order to interpolate relative sample values. The S1P standard curve (below) shows an 8 point S1P curve that was generated using non-linear regression analysis with GraphPad Software. A semi log [Sigmoidal dose-response (variable slope)] analysis was utilized. For best results, constrain the standard curve top & bottom using the 0 µM S1P & Blank controls. The S1P concentration, for all samples, should include the dilution factor utilized in step 3 of reagent preparation.



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

If you are testing tissue homogenate or cell lysate samples the S1P standard curve should be graphed in pmol/well S1P (see table). The interpolated sample values should then be normalized with grams of total protein or mgs of tissue.

Precautions

Assay Performance

For best results, please follow the protocols provided. Not following the instructions may result in suboptimal performance of the kit and failure to produce accurate data. All sample types must be diluted in the Delipidized Serum provided in the kit.

Health Hazard Data

The kit contains Delipidized Serum. This reagent should be handled as a potentially bio-hazardous 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 1/2 (HIV) and Hepatitis C Virus (HCV), non-reactive to Human Immunodeficiency Virus 1 (HIV1), Hepatitis B surface antigen (HBsAg) and Sexually Transmitted Diseases (RPR). Since no test method can offer complete assurance that infectious agents are absent, the Delipidized Serum should be handled following all universal precautions.

Research Use Only.

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