Echelon Biosciences Inc.

Lysophosphatidic Acid (LPA) Assay Kit II

K-2800S (96 tests)

Support: echelon@echelon-inc.com

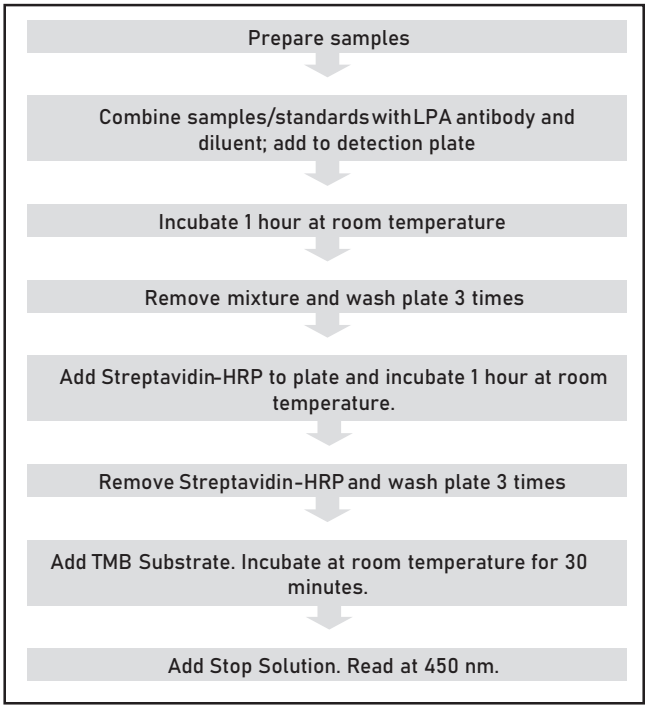
Materials Provided

Catalog #	Description	Amount
K-2801S	Biotinylated Anti-LPA Antibody	< 100 µL
K-2805L	Lysophosphatidic Acid (LPA C18:3) Standard	238 µg
K-2807S	LPA Detection Plate	1 Plate
K-SEC3h	Streptavidin HRP	80 µL
K-PBST4	Sample Diluent	10 mL
K-PBST2	10X PBS-T Buffer	20 mL
K-TMB1	TMB Substrate	12 mL
K-STOPt	1N Sulfuric Acid (H_2SO_4)	8 mL
---	Microtiter plate seal	2 Seals

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

Quick Protocol



Storage: Upon receipt, store Part 1 (kit box) at 4 °C and Part 2 at -80 °C. The LPA standard is not stable at temperatures > -70 °C. Under proper storage, this product is stable 6 months from date of receipt. Opened and reconstituted solutions are less stable.

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Ph: 801-588-0455
Fax: 801-588-0497
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Background

Lysophosphatidic Acid (LPA) is a serum-derived phospholipid involved in diverse cellular processes such as cell proliferation, chemotaxis, platelet aggregation, wound healing, angiogenesis, tumor invasion, and smooth muscle contraction. Recent research indicates LPA may play a significant role in the pathophysiology of cancer and may be used as a biomarker for ovarian cancer.

Assay Design

The Lysophosphatidic Acid (LPA) Assay Kit II is an enzyme-linked immunoassay designed for the in vitro measurement of LPA in biological samples. The assay is a competitive ELISA in which the colorimetric signal is inversely proportional to the amount of LPA present in the sample (increased LPA = reduced assay signal).
Briefly, the samples are pre-mixed with the biotinylated anti-LPA antibody and a sample diluent before the mixtures are added to the LPA coated detection plate for competitive binding.
Streptavidin-HRP and colorimetric detection are used to detect the amount of biotinylated anti-LPA bound to the plate. The concentration of LPA in the sample is determined using a standard curve of known amounts of LPA. This assay is read at 450 nm and requires 3 hours to run.

Assay Range 0.064 µM to 1,000 µM

Sensitivity The limit of detection (LOD), defined as the lowest amount of LPA in a sample detectable from zero, was calculated at 0.12 µM. The limit of quantification (LOQ) or the lowest concentration detected within the linear range of the curve was calculated at 0.024 µM. The LOD and LOQ values are from an average of 4 experiments.

Specificity No significant cross reactivity was observed against any lipid tested. The lipids we selected for testing were related in structure and signaling pathway. For a complete list of the lipids tested please contact customer service. As it is impossible for us to run a complete cross-reactivity and interference test on the assay, cross reaction may still exist.

Assay Precision The assay's precision was determined with 4 human serum samples and 2 human plasma samples. The experiments were run in triplicate, 4 times, with two lots of kits.
Intra-assay (precision within an assay): 6.5-8.7%
Inter-assay (precision between assays): 8.9-15.33%

Sample Type This kit detects LPA from human or animal in serum, plasma and tissue homogenate.

Sample Volume 20 µL/ sample is needed for duplicate data points.

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.

Assay Notes

1. Ensure samples are free from debris before addition to plate.
2. Ensure all reagents are warmed to room temperature before use. Failing to do so might result in edge effects and increased CV's.
3. Samples/standards: Antibody: Sample Diluent mixing ratio, in step 5, is 1:9:10. Volumes can be adjusted. Sample Diluent must be added last. Mixture is mixed by vortex for 1-2 seconds to achieve the optimal assay reproducibility.
4. When analyzing biologic samples we advise running a known normal (low) LPA sample and a disease (high) LPA sample in conjunction with your unknown samples. These will serve as positive and negative controls to distinguish between normal healthy samples and disease samples.
5. The antibody used in this assay has varied binding affinity to different LPA species. The order of the antibody affinity to different LPA species are C18:3 > C18:2 > Mixed LPA > C20:4 > C14:0 > C18:1 > C16:0 > C18:0. Mixed LPA contains LPA C16:0, C18:0, C18:1, C18:2 and C20:4 at 2.8:1:1.5:2.6:3 concentration ratios.
6. This assay is optimized for human serum and plasma. The assay can be used for LPA detection in animal samples but this application has not been optimized. Sample dilution may need to be optimized and is highly recommended before running a panel of samples.
7. The LPA Assay Kit II has been optimized for the detection of LPA in serum and plasma samples. Sample optimization is highly recommended for tissue homogenate samples.

Protocol for LPA Detection

Please read this entire section and the assay notes section before beginning the 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, prepare samples (see support protocols) and place the Streptavidin HRP (K-SEC3h), Biotinylated Anti-LPA Antibody (K-2801S), and LPA Standard (K2805L) on ice. Allow the remaining kit components to warm to room temperature before use.

1. Prepare 1X PBST by diluting the bottle of 10X PBS-T Buffer (K-PBST2) with 180 mL deionized water. Store prepared 1X PBST at room temperature.

2. Reconstitute the LPA Standard (K-2805L)
   a. For serum or plasma samples, reconstitute the LPA Standard (K-2805L) with 50 µL of 1X PBST (step 1) for 1,000 µM solution. Vortex to mix and keep at room temperature. The reconstituted LPA standard can handle at least one freeze-defrost cycle and is stable for 6 months at < -20°C.
   b. For tissue homogenate samples, reconstitute the LPA Standard (K-2805L) with 50 µL of tissue homogenate buffer (see buffer recipe in support protocols) for 1,000 µM solution. Vortex to mix. Keep at room temperature. If samples have been prepared in two different buffers then a separate standard curve will need to be made for each sample buffer used. An extra LPA standard can be purchased under cat# K-2805L. The standards stability in homogenate buffer is unknown.
3. Vortex the reconstituted LPA standard (step 2) and prepare the 7 LPA standard dilutions in 1X PBST (step 1) or homogenate buffer as described in Table 1.

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4. Add 7 mL 1X PBST (step 1) directly into the Biotinylated Anti-LPA Antibody bottle (K-2801S). Mix well. Place at room temperature and immediately proceed to the next step. **This reagent is not stable at the working concentration and should be prepared immediately before use.** The diluted antibody can handle at least one freeze – defrost cycle and is stable for 7 days storage at -20 °C. A change in the assays signal may be observed.

5. Combine the 7 LPA standard (step 3), or samples, with the diluted antibody (step 4) and Sample Diluent (K-PBST4) in 1.5 mL polypropylene tubes in the following order. If desired, a 96-well incubation plate can be used instead of the 1.5 mL polypropylene tubes.
   a. First, add 135 µL of the diluted antibody (step 4) into each tube. For negative control, add 135 µL of 1X PBST (step 1).
   b. Second, add 15 µL of the LPA standards (step 3) or prepared samples into appropriate tubes. For positive and negative controls, add 15 µL of the solution used to dilute the standards. This should be 1X PBST (step 1) and/or homogenate buffer.
   c. Third, add 150 µL of Sample Diluent (K-PBST4) to all tubes.
      i. Ensure pipette tip does not contact the sample/antibody mixture to avoid cross contamination between samples.
      ii. If precipitation is present in the Sample Diluent (K-PBST4), vortex vigorously until precipitate is dissolved.
   d. Vortex each tube for 1-2 seconds to mix. Incubate tubes 15-20 minutes at room temperature.
6. Remove the LPA Detection Plate (K-2807S) from plastic bag. Add 100 µL/well of the prepared standards, controls, and samples (step 5) according to plate layout. according to the "Suggested LPA Detection Plate Layout", see Table 2 below. For best results, prime the pipette 3-6 times before transferring. Cover the plate with a plate seal and incubate at room temperature for 1 hour.
7. After incubation, discard solutions from the LPA Detection Plate and wash with 200 µL/well of the 1X PBST (step 1) three times. Ensure all wash buffer is removed before proceeding to the next step.
8. Dilute 60 µL of the Streptavidin HRP (K-SEC3h) with 12 mL of the 1X PBST (step 1). Mix well. If you are running experiments over multiple days, only prepare what is needed for the day. Prepare immediately before use. The stock Streptavidin HRP (K-SEC3h) can withstand 3 freeze-defrost cycles and should be stored at -20°C to -80°C between uses.
9. Add 100 µL/well of the diluted Streptavidin HRP solution (step 8) to the LPA Detection Plate. Cover with new plate seal and incubate at room temperature for 1 hour.
10. After incubation, discard solutions from the LPA Detection Plate and wash with 200 µL/well of the 1X PBST (step 1) three times. Ensure all wash buffer is removed before proceeding to the next step.
11. Add 100 µL/well of the TMB Substrate (K-TMB1) and incubate for 30 minutes in a dark location. Then add 50 µL/well 1N Sulfuric Acid (K-STOPt) to the Microtiter Plate to stop the reaction. Read Microtiter Plate absorbance at 450 nm.

Table 1, LPA Standards

LPA Concentration	Amount of 1,000µM LPA Stock (Step 3) or Previous Dilution	Amount of 1X PBST or homogenate buffer
1000 µM	45 µL of 1000µM LPA Solution	None
200 µM	5 µL of 1000µM LPA Solution	20 µL
40 µM	5 µL of 200µM LPA Solution	20 µL
8 µM	5 µL of 40µM LPA Solution	20 µL
1.6 µM	5 µL of 8µM LPA Solution	20 µL
0.32 µM	5 µL of 1.6µM LPA Solution	20 µL
0.064 µM	5 µL of 0.32µM LPA Solution	20 µL
0 µM (Pos. Ctrl)	0 µL	25 µL

Table 2, Suggested LPA

	1	2	3	4	5	6	7	8	9	10	11	12
A	1000 µM LPA	1000 µM LPA	Sample #1	Sample #1	Sample #8	Sample #8	Sample #16	Sample #16	Sample #24	Sample #24	Sample #32	Sample #32
B	200 µM LPA	200 µM LPA	Sample #2	Sample #2	Sample #9	Sample #9	Sample #17	Sample #17	Sample #25	Sample #25	Sample #33	Sample #33
C	40 µM LPA	40 µM LPA	Sample #3	Sample #3	Sample #10	Sample #10	Sample #18	Sample #18	Sample #26	Sample #26	Sample #34	Sample #34
D	8 µM LPA	8 µM LPA	Sample #4	Sample #4	Sample #11	Sample #11	Sample #19	Sample #19	Sample #27	Sample #27	Sample #35	Sample #35
E	1.6 µM LPA	1.6 µM LPA	Sample #5	Sample #5	Sample #12	Sample #12	Sample #20	Sample #20	Sample #28	Sample #28	Sample #36	Sample #36
F	0.32 µM LPA	0.32 µM LPA	Sample #6	Sample #6	Sample #13	Sample #13	Sample #21	Sample #21	Sample #29	Sample #29	Sample #37	Sample #37
G	0.064 µM LPA	0.064 µM LPA	Sample #7	Sample #7	Sample #14	Sample #14	Sample #22	Sample #22	Sample #30	Sample #30	Sample #38	Sample #38
H	Pos. Ctrl.	Pos. Ctrl.	Neg. Ctrl.	Neg. Ctrl.	Sample #15	Sample #15	Sample #23	Sample #23	Sample #31	Sample #31	Sample #39	Sample #39

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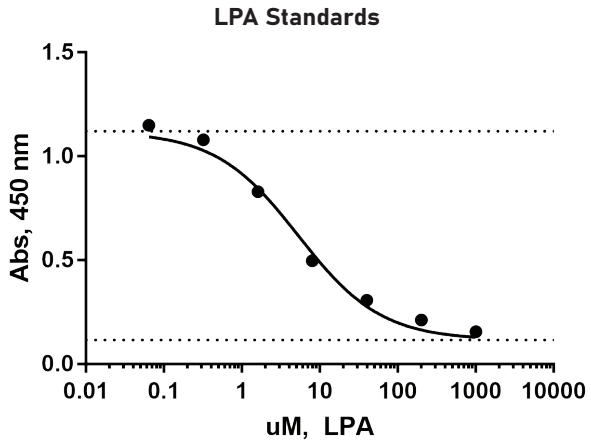
Quantification of Samples



Generate a best fit curve for the LPA standards in order to interpolate relative sample values. The example LPA standard curve, above, shows a 7 point LPA curve that was generated using non-linear regression analysis with Graphpad Software. A semi log [Sigmoidal dose-response (variable slope)] (4 paramter) analysis was utilized. For best results, constrain the standard curve top & bottom using the positive and negative controls. If samples have been diluted, the dilution factor must be multiplied back into the concentration calculated.

Support Protocols

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.

Preparation of Serum

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 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,

Preparation of Tissue Homogenate

For tissue homogenate and cell culture samples we recommend > 30 µg of total protein/well. 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.

Procedure

1. Homogenized tissues were tested in the following methods.
   a. By sonication in the following homogenization buffer: 20 mM Tris-HCl, pH 7.4; 20% glycerol; 1 mM β-mercaptoetha-nol; 1 mM EDTA; 1 mM Naorthovanadate; 15 mM NaF; 1 mM PMSF; protease inhibitor cocktail (Sigma); 0.5 mM deoxy-pyridoxine; 40 mM β-glycerophosphate. Centrifuge 5 minutes at 5000 x g, 2 - 8°C. The supernatant is then removed and assayed.
   b. By freeze-defrost cycles in 1X PBS: Rinse tissue in 1X PBS and store overnight in 1X PBS. Perform two freeze-thaw cycles. Centrifuge 5 minutes at 5000 x g, 2 - 8°C. The supernatant is then removed and assayed.
2. Total protein concentration should be measured and the samples diluted with the homogenate buffer to the same protein concentration before being added to the assay.
3. The homogenates can be stored frozen at -80°C until analysis. Avoid repeat freeze-thaw cycles. Centrifuge the sample again after thawing and before adding to the assay.

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References

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Related Products

Products	Catalog Number
Assays and Enzymes	
Autotaxin Enzyme, active	E-4000
Autotaxin Activity Assay	K-4100
Autotaxin Inhibitor Screening Kits	K-4200, K-4200HTS
Autotaxin ELISA	K-5600
Antibodies	
Anti-LPP3-c-cyto Rabbit Polyclonal Antibody	Z-R018
Anti-LPA Mouse Monoclonal Antibody	Z-P200
Fluorescent ATX Substrates	
FS-3 (lysoPLD)	L-2000
ATX-red	L-2010

Products	Catalog Number
Lipids	
LPA Biotin	L-012B
LPA C18:1, LPA C18:2, LPA C18:3	L-0181, L-0182, L-0183
Ether-linked LPA	L-0186
LPA C20:0, LPA C20:4	L-0200, L-0204
Inhibitors	
XY-14 (Lipid Phosphate Phosphatase 1 (LPP1) inhibitor)	L-9218
LPA3 (edg 7) selective ligands	L-9118, L-9318, L-9418, L-9518
BrP-LPA	L-7416
ATX Inhibitors	B-0701, B-0702, L-3223, L-3282, L-7118, L-7218

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Fax: 801-588-0497
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