Echelon Biosciences Inc.

Phosphatidylethanol (PEth) ELISA Kit

K-5000 (96 tests)

Support: echelon@echelon-inc.com

Description: 96-well ELISA for Detection and Quantification of PEth

Materials Provided

Catalog #	Description	Amount
K-5001	PEth Detection Plate	1 plate
K-5002	PEth Standards (A – F)	6 vials, 400 µL each
K-5003	Antibody Stabilizer	1 vial, 1.2 mL
K-5004	Anti-PEth Antibody	20 µL
K-5005	Sample Buffer	10 mL
K-SEC5	Secondary Detector	50 µL
K-PTAB	PBS Tablet	1 Tablet
K-PBST2	10x PBS-T Buffer	20 mL
K-TMB1	TMB Solution	12 mL
K-STOPt	1 N H_2SO_4 Stop Solution	10 mL
Incubation Plate	Yellow 96-well polypropylene U-bottom plate	1 plate
Plate Sealers	Clear acetate sheet, 1 side adhesive	2 seals

Quick Protocol

Prepare all reagents, standards, and samples

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Combine PEth standards and samples with PEth antibody in the Incubation Plate and incubate for 1.5 hours at RT

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Transfer incubation mixtures to the Detection Plate and incubate for 45 minutes at RT

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Wash the wells 4 times with 200 µL/well PBST

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Add 100 µL/well of Secondary Detector and incubate for 45 minutes at RT

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Wash the wells 4 times with 200 µL/well PBST

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Add 100 µL/well TMB and develop for 30 minutes at RT

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Add 50 µL/well Stop Solution and read absorbance at 450 nm.

Additional Materials Provided by User

- Extracted PEth samples (See Support Protocol for PEth Extraction from Blood at the end of this document)
- Buffers and solvents for PEth extractions
- 450 nm absorbance plate reader

Storage

The kit comes in two parts with different storage requirements. Upon receipt store PEth ELISA Kit Part 1 at 4°C and PEth Standards Part 2 at -20°C. Store reagents as indicated in the protocol. Allow the reagents to warm to room temperature before opening the vials.

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Background

Ethanol (EtOH) has multiple effects on humans and other animals. Alcohol addiction and organ damage are active areas of research with a need for research reagents and tools.[1] Phosphatidylethanol (PEth) is a direct metabolite of EtOH and has been described as a long-lived biomarker for alcohol ingestion.[2,3] It is possible that PEth is involved in the neurological[4], protective[5], and harmful[6] effects of alcohol, but the mechanisms are still being explored.

Assay Design

Echelon's PEth ELISA assay is a competitive ELISA in which the signal is inversely proportional to the amount of PEth in the sample. Once PEth has been generated in vitro or extracted from erythrocytes or serum, the sample is incubated with an anti-PEth antibody and then added to a PEth-coated plate for competitive binding. A peroxidase-linked secondary detector and colorimetric substrate is used to detect the amount of anti-PEth antibody bound to the plate. The colorimetric signal is read at absorbance 450 nm and is inversely proportional to the amount of PEth in the sample.

Assay Notes

1. Incubation times are optimized. Deviation will result in a shift of the standard curve and an increase or decrease of signal.
2. Never let the detection plate dry out after the assay has started. Always have the next solution ready to add before discarding the current one.
3. Use caution when using acid stop solution.

Assay Protocol

Please read this entire section and Assay Notes before beginning .

Reagent Preparation

1. PBS Buffer
   Prepare PBS buffer by adding 200 mL dH2O to provided PBS tablet (K-PTAB). PBS is stable at room temperature.
2. PBS 10% Antibody Stabilizer
   Prepare PBS 10% Antibody Stabilizer by adding 1 mL Antibody Stabilizer (K-5003) to 9 mL PBS. Vortex briefly.
3. Wash Buffer
   Prepare Wash Buffer by diluting 20 mL of the 10x PBS-T Buffer (K-PBST2) with 180 mL dH2O. Wash Buffer is stable at room temperature.
4. PEth Standards and Samples
   Reconstitute or dilute PEth samples in Sample Buffer (K-5005). PEth standards (K-5002 A - F) and samples should be warmed in a water bath to 35 – 40°C, vortexed, and used between 25 – 35°C.
5. Anti-PEth Antibody
   Dilute Anti-PEth Antibody (K-5004) 1:600 by adding 10 µL Anti-PEth to 6 mL PBS 10% Antibody Stabilizer for the entire plate.

ELISA Set up and Incubation

We suggest that PEth samples, controls, and standards be run in duplicate or triplicate. The protocol is written for triplicate points. Volumes will need to be adjusted if duplicate points are used.

1. Combine samples and PEth standards with diluted anti-PEth antibody in the colored incubation plate (see Table 1).
   a. Add 175 µL of PEth Standard A – F to Rows A – F in Column 1
   b. Add 175 µL Sample Buffer (K-5005) to well G1 for a No Lipid Control.
   c. Add 350 µL of Sample Buffer (K-5005) to well H1 for a Blank Control.
   d. Add 175 µL of PEth individual Samples to Columns 2, 3, and 4.
   e. Add 175 µL of diluted Anti-PEth Antibody (K-5004) to all the wells except Blank control well H1.
   Only part of the incubation plate should be utilized. Each well in the incubation plate, after incubation, will be transferred to triplicate wells of the PEth Detection Plate (K-5001).
2. Cover incubation plate with acetate plate sealer and incubate on plate shaker for 1.5 hours at room temperature.
3. Transfer 100 µL of mixture from the incubation plate to triplicate wells of the PEth Detection Plate (K-5001). See Table 2. Cover plate with acetate plate sealer and incubate on plate shaker for 45 min at room temperature.
4. Discard contents and wash the wells 4 times with 200 µL/well Wash Buffer.
5. Dilute Secondary Detector (K-SEC5) 1:300 by adding 40 µL to 12 mL PBS for the entire plate. Mix well and add 100 µL/well of diluted Secondary Detector to each well. Cover and incubate on a plate shaker for 45 min at room temperature.
6. Repeat wash step # 4.
7. Add 100 µL of TMB solution (K-TMB1) to each well. Allow color to develop for 30 minutes in dark (or cover plate with aluminum foil). Watch for blue color development. DO NOT overdevelop.
8. Stop color development by adding 50 µL 1 N H_2SO_4 stop solution (K-STOPt) to each well when the color is clear to light blue in the 400 µM standard well and has turned medium to dark blue in the No Lipid control wells. Blue color will change to yellow color upon addition of stop solution. Eliminate air bubbles present in the wells before reading the plate.
9. Read plate absorbance at 450 nm on a plate reader

Results

Cellular PEth quantities can be estimated by comparing the values from the wells containing PEth extraction products to the values in the standard curve. Plot the absorbance values obtained vs. amount of PEth per standard to generate a standard curve. Determine where the values obtained from the PEth extractions lie on the curve to obtain a measure of PEth in your samples. The standard curve belwo was generated using non-linear regression analysis with GraphPad Prism software. A sigmoidal dose-response variable slope (four-parameter) curve fit was utilized.

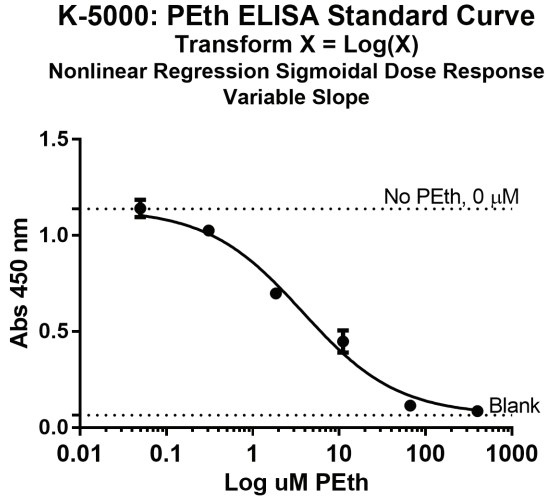

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Table 1, Suggested Incubation Plate Layout

	K-5002 PEth Standards and Controls	Samples			Empty							
	1	2	3	4	5	6	7	8	9	10	11	12
A	Standard A K-5002a 400 µM (Red)	Sample 1	Sample 9	Sample 17	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-
B	Standard B K-5002b 66.7 µM (Orange)	Sample 2	Sample 10	Sample 18	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-
C	Standard C K-5002c 11.1 µM (Yellow)	Sample 3	Sample 11	Sample 19	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-
D	Standard D K-5002d 1.85 µM (Green)	Sample 4	Sample 12	Sample 20	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-
E	Standard E K-5002e 0.31 µM (Blue)	Sample 5	Sample 13	Sample 21	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-
F	Standard F K-5002f 0.05 µM (Purple)	Sample 6	Sample 14	Sample 22	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-
G	Sample Buffer (K-5005) No Lipid	Sample 7	Sample 15	Sample 23	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-
H	Sample Buffer (K-5005) Blank	Sample 8	Sample 16	Sample 24	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-	-Empty-

Table 2, Suggested Detection Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	400 µM	400 µM	400 µM	Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17
B	66.7 µM	66.7 µM	66.7 µM	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18
C	11.1 µM	11.1 µM	11.1 µM	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19
D	1.85 uM	1.85 uM	1.85 uM	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20
E	0.31 µM	0.31 µM	0.31 µM	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21
F	0.05 µM	0.05 µM	0.05 µM	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22
G	0 µM	0 µM	0 µM	Sample 7	Sample 7	Sample 7	Sample 15	Sample 15	Sample 15	Sample 23	Sample 23	Sample 23
H	-Blank-	-Blank-	-Blank-	Sample 8	Sample 8	Sample 8	Sample 16	Sample 16	Sample 16	Sample 24	Sample 24	Sample 24

Support Protocol

PEth in blood is found primarily associated with erythrocytes or red blood cells (RBCs).[7] A number of approaches to PEth extraction and analysis (mostly based on Radin,[8] and using mass spectrometry analysis) have been reported. Following are three methods with corresponding citations published by independent laboratories. Users are encouraged to begin with these protocols, and develop and optimize a method in their own lab. Echelon has not validated these protocols with the K-5000 PEth ELISA.

Protocol 1

1. 0.9 mL of blood (heparin or EDTA anticoagulated) is added to 4 mL propan-2-ol (while vortex mixing), then 6 mL of hexane is gradually added.
2. Mix sample for 10 min
3. Centrifuge the sample for 10 min at 1,500 x g.
4. Collect the upper phase supernatant into a new tube and dry with a stream of inert gas, such as nitrogen or argon.

Protocol 2

1. 100 µL borate buffer, pH 9 (0.63 M), and 800 µL isopropanol is added to 300 µL blood.
2. After vortexing, 1,200 µL n-hexane is added, and the mixture is agitated for an additional 10 min
3. Centrifuge the sample for 5 min at 16,000 x g.
4. Collect the supernatant and evaporate to dryness under a nitrogen stream at 40 °C.

Protocol 3

1. 100 µL blood is added stepwise to 600 µL isopropanol under constant vortex-mixing.
2. Samples are gently mixed for 10 min and 2×450 µL heptane is added with mixing after each addition.
3. Samples are centrifuged for 10 min, 2,000 x g at 4 °C.
4. The clear supernatant is transferred to new glass tubes and evaporated to dryness under a stream of nitrogen gas at 30 °C using a metal block.

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References - Background

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References - Product Publications

1. Eby JM, Majetschak M. Effects of ethanol and ethanol metabolites on intrinsic function of mesenteric resistance arteries. PLoS ONE. 2019;14(3):e0214336.

Related Products

Catalog #	Products
PEth Binding Reagents	
Z-PETH	Anti-Phosphatidylethanol Antibody
P-BPEth	Phosphatidylethanol (PEth) Beads
Lipids	
L-6017	17:0, 18:1 Phosphatidylethanol
L-6018	18:1,18:1 Phosphatidylethanol
L-6019	16:0,18:1 Phosphatidylethanol
L-6020	16:0, 18:2 Phosphatidylethanol
L-60F18	BODIPY-FL-Phosphatidylethanol
L-60N16	NBD-Phosphatidylethanol
L-60B16	C12-Biotin, 16:0-Phosphatidylethanol
L-60B18	C12-Biotin, 18:0-Phosphatidylethanol
L-6051	d5-POPEth (deuterated)
L-6052	d5-PLPEth (deuterated)
L-6053	d5-SOPEth (deuterated)
L-6054	d5-SLPEth (deuterated)
L-6055	d5-PAPEth (deuterated)

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