Echelon Biosciences Inc.

Phosphatidylethanol (PEth) ELISA

K-5500 (96 tests)

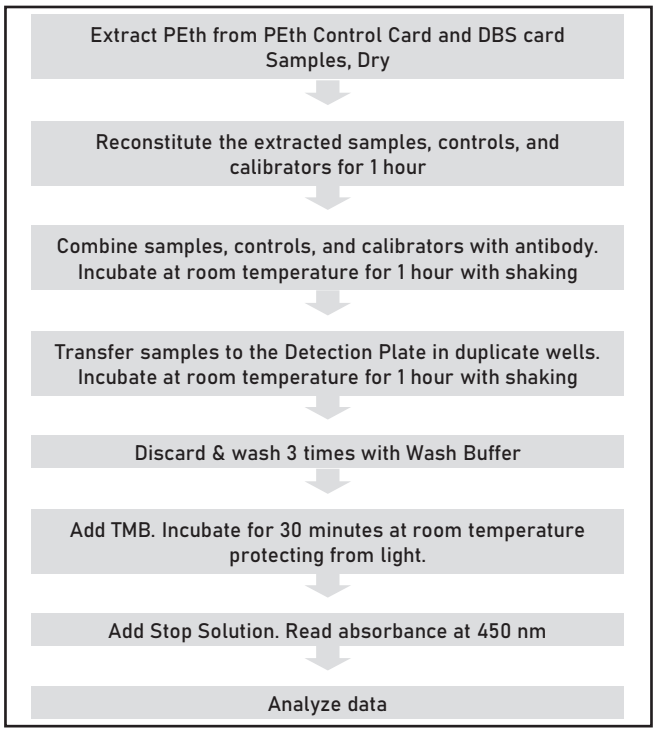
Support: echelon@echelon-inc.com

Description: ELISA to detect the alcohol biomarker, PEth, extracted from dried blood spot (DBS) cards

Materials Provided

Catalog #	Description	Amount
K-5001	Detection Plate	1 plate
K-5503	Sample Diluent	1 bottle
K-5504	Antibody Diluent	1 bottle
K-5505	Anti-PEth Antibody	1 vial
K-PBST2	10x PBS-T	1 bottle
K-TMB1	TMB Solution	1 bottle
K-STOPt	Stop Solution (1 N H_2SO_4)	1 bottle
------	Pre-incubation Plate (Yellow)	1 plate
------	Acetate Plate Sealer	2 seals
K-5506	Zero Calibrator	1 vial
K-5507	Low Calibrator	1 vial
K-5508	High Calibrator	1 vial
K-5510	PEth Control Card	1 card

Quick Protocol



Additional Materials Provided by User

- Speedvac (optional)
- Plate reader for reading absorbance at 450 nm
- Plate shaker
- Methanol (for Sample extraction)
- 3.2 mm round hole punch

Storage

Upon receipt, store the kit at 4 °C. Under proper storage conditions, this product is stable for at least 6 months from date of receipt. Opened and reconstituted reagents are less stable. Refer to assay notes for additional storage information.

This product is intended for use with samples extracted from dried blood spot (DBS) cards. We recommend using only the procedure outlined in this protocol.

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Background

Phosphatidylethanol (PEth or Ptd-OH) is a direct biomarker of alcohol ingestion formed in the blood only when ethanol has been consumed. It is specific and can report previous alcohol consumption beginning a few hours after a drinking event and continuing 2-3 weeks later. Sensitive detection by mass spectrometry using whole blood or dried blood spot (DBS) cards is becoming generally accepted as the gold-standard for detecting past alcohol consumption. The Echelon PEth ELISA is the first commercially available immunoassay for detection of PEth in blood.

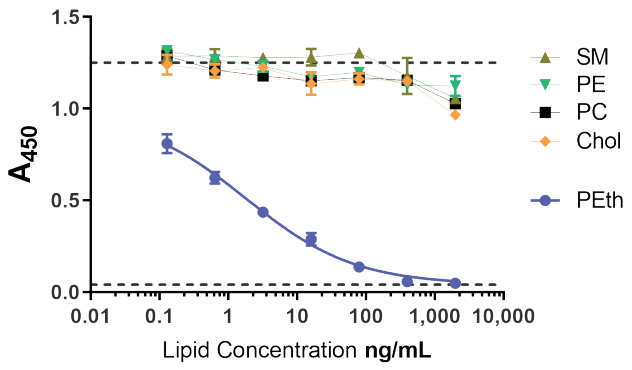
Assay Design

The assay is a competitive immunoassay where PEth present in a sample extract competes with PEth on a plate for binding to a specific HRP-conjugated antibody. Thus, the assay signal is inversely proportional to the amount of PEth present in the sample. This assay is designed for dried blood spot card samples and uses 5 small 3.2 mm (1/8 inch) punches for running duplicate or triplicate measurements. The antibody recognizes all major PEth molecular species, providing researchers a quick and robust method to screen for positive/negative PEth in whole blood spotted DBS cards.

Assay Notes

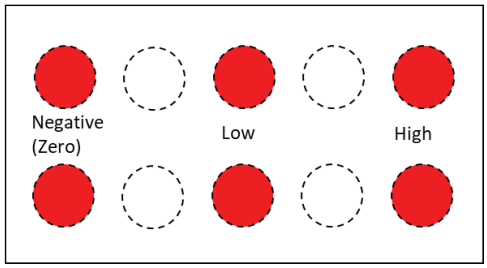
1. Use Nanopure or equivalent laboratory grade water free of contaminants.
2. Bring all reagents to room temperature before use, except the Anti-PEth Antibody (K-5505) which should remain at 4 °C or on ice until use.
3. If a plate shaker is not available, tap plate to mix after all reagents are added. The plate can be incubated without shaking but the user should expect a higher coefficient of variation.
4. Incubation times are optimized. Deviation from these times may result in aberrant signals.
5. This assay is optimized for measuring PEth from whole blood spotted on DBS cards. Serum and plasma specimens are NOT compatible with this assay. If the specimen is collected in a tube via venipuncture, we recommend pipetting 50 µL of whole blood on a DBS card, letting the sample dry 3+ hours before punching and extracting as described in the extraction protocol. Storage of whole blood prior to spotting DBS cards can affect the results. For best results, avoid freezing or storage of any whole blood samples over 1-2 weeks at 4°C.
6. The antibody in this assay does not cross react with other major lipid species found in blood (Figure 1).

Figure 1. Assay specificity with other major lipids found in red blood cell erythrocytes.



7. The Anti-PEth Antibody (K-5505) is diluted 1:150 fold with K-5504 Antibody Diluent. Use 50 uL Antibody with 7.5 mL Diluent for a full plate.
8. The ratio of sample or calibrator to antibody solution is 1:1 (Step 5 of ELISA Protocol). The total volume can be adjusted as needed for single, duplicate, or triplicate wells, but the ratio of sample to antibody should remain 1:1.
9. PEth Immunoassay Standards:
   Calibrators: The K-5506, K-5507, and K-5508 Calibrators were extracted from whole blood samples spotted on DBS cards.
   PEth Control Card: The K-5510 Control Card contains three PEth control standards that are to be run in parallel to the user's samples. These standards should experience the same treatment as the user's samples and, are therefore the best reference controls for the assay.

K-5510 "PEth Control Card"



PEth Extraction Protocol

Please read the entire PEth Extraction & ELISA Protocols before beginning the experiment.

1. Turn the DBS cards over and visually select a punch target completely saturated with blood. Using the hole puncher, punch five, 3.2 mm circles for each control and sample.
2. Place the punched circles into properly labeled polypropylene microcentrifuge tubes, and add 500 µL methanol. Small lab forceps or tweezers may be helpful.
3. Cap the tubes, vortex 1 min, and incubate for 1 hour at room temperature (20-22 °C).
4. Vortex the tubes again for 1 min at the end of the incubation then centrifuge at 4,000 x g for 3 min. Transfer 400 µL of the methanol extract to a fresh polypropylene tube without disturbing the punches.
5. Completely evaporate the methanol from the extracted samples and controls. This can be accomplished in a speed-vacuum for 60 min without heating, OR allowing sample tubes to stand open overnight in a fume hood, biological safety cabinet, or protected clean environment, OR other similar drying method avoiding heating. Ensure the samples are dry. Analyze directly by ELISA or store dried samples at -20°C until use.

ELISA Protocol

This protocol is written for duplicate samples. Please refer to Assay Note #8 if other than duplicates are run.

1. Bring all reagents to room temperature before use, except the Anti-PEth Antibody (K-5505) leave this at 4 °C or keep on ice until diluted.
2. Reconstitute the dried, extracted controls, samples and calibrators (K-5506, 5507, & K-5508) with 300 µL Sample Diluent (K-5503). Vortex for 1 minute, let sit at room tempera-

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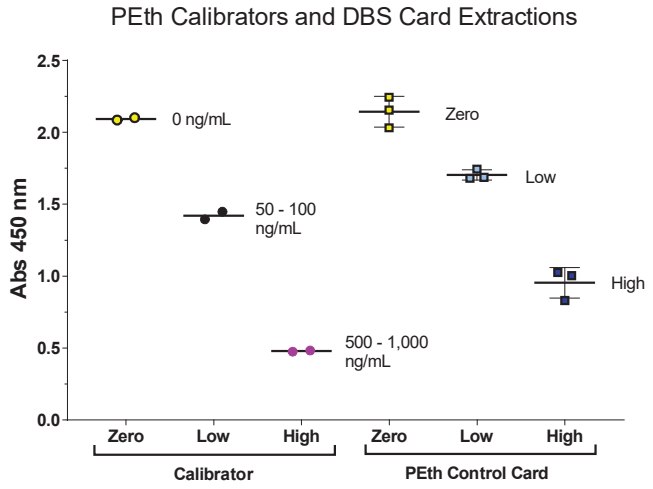
ture for 1 hour, then vortex again for 1 minute to fully reconstitute the samples and calibrators.

3. Prepare Wash Buffer by adding 20 mL 10x PBS-T (K-PBST2) to 180 mL H2O. Mix well. Store at room temperature.
4. Dilute Anti-PEth Antibody (K-5505) by adding 50 µL to 7.5 mL Antibody Diluent (K-5504).* Mix gently but thoroughly. Keep at room temperature until use. *See Assay Note 7
5. Combine the reconstituted samples, controls, and calibrators (step 2) with the diluted Anti-PEth Antibody (step 4) in the Pre-Incubation Plate as follows:
   a. Vortex the reconstituted samples and calibrators.
   b. For Blank (Well A1), add 120 µL Sample Diluent (K-5503), and 120 µL Antibody Diluent (K-5504).
   c. For "Ab Only Control" (Well B1), add 120 µL Sample Diluent (K-5503).
   d. For Well C1, add 120 µL of Zero Calibrator (K-5506).
   e. For Well D1, add 120 µL of Low Calibrator (K-5507).
   f. For Well E1, add 120 µL of High Calibrator (K-5508).
   g. Add 120 µL of reconstituted PEth controls and samples to the remaining wells of the first six columns of the Pre-incubation plate (see layout).
   h. Add 120 µL of the diluted Anti-PEth Antibody (step 4) into all remaining wells except Blank (Well A1).

   (See diagram below for a suggested plate layout)
6. Seal the plate with an acetate plate sealer and incubate with gentle shaking for 1 hour at room temperature.
7. After incubation, use a multichannel pipette to pre-mix solutions three times before transferring 100 µL to duplicate wells of the Detection Plate (K-5001). Cover plate with an acetate plate seal and incubate on plate shaker for 1 hour at room temperature.
8. After incubation, discard solutions and wash wells three times with 200 µL/well of Wash Buffer (Step 3).
9. Add 100 µL/well TMB substrate (K-TMB1). Cover plate with acetate plate sealer and incubate for 30 min in the dark. Do not shake.
10. Carefully remove the acetate plate sealer. Add 50 µL/well 1N H2SO4 (K-STOPt) to stop reaction.
11. Read the plate at 450 nm. Analyze according to user's preferred method. Example of Calibrators and Control Card Sample competition is shown in Figure 3.

Figure 3. Example of Calibrators and Control Samples Competition in PEth ELISA (K-5500)



Suggested Incubation Plate Layout

Row	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
A	Blank	Additional Samples				
B	Ab Only Control					
C	Zero Calibrator					
D	Low Calibrator					
E	High Calibrator					
F	Zero Control					
G	Low Control					
H	High Control					Additional Samples

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References - Background

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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)

Please visit our website at www.echelon-inc.com for more lipid, antibody, and ELISA products.

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