



PROVIDER OF CE-CERTIFIED LC-MS/MS DIAGNOSTIC KITS

INSTRUCTIONS FOR USE

FOR THE IN VITRO DETERMINATION OF PETH 16:0/18:1 IN  
SERUM AND WHOLE BLOOD

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## PHOSPHATIDYLETHANOL, PETH, LC-MS/MS KIT

**Art. No. 50-2002, 400 analyzes**

**US Pat. 9499572, 9784701**

**EPO 2992334**

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### 1. INTENDED USE

For the in vitro determination of Phosphatidylethanol (PEth) 16:0/18:1 in whole blood

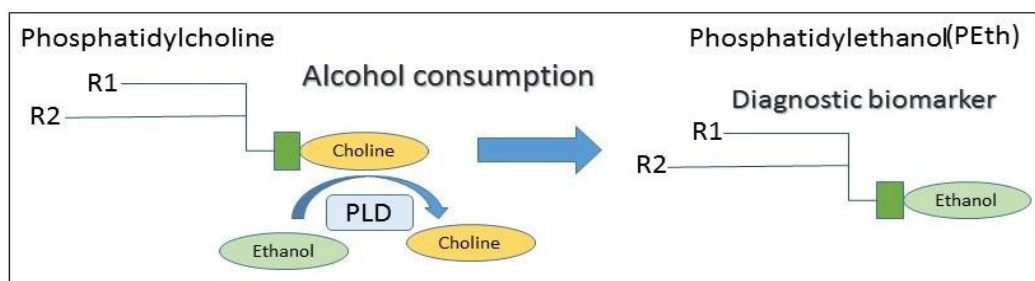
The described LC-MS/MS application is intended for the quantitative determination of phosphatidylethanol (PEth) in whole blood.

For in vitro diagnostics use.

### 2. INTRODUCTION

Phosphatidylethanol (PEth) in whole blood is a biomarker for alcohol consumption.

PEth is an unnatural phospholipid that is formed only in the presence of ethanol, giving a theoretical diagnostic specificity of 100% as a biomarker for alcohol consumption. The principle for in-vivo generation of PEth depends on rearrangement of phosphatidylcholine in presence of phospholipase D. PEth-16:0/18:1 is the most abundant individual form of PEth-homologues and is used in quantitative LC-MS/MS analysis of blood from patients to estimate the level of alcohol consumption. There is a direct correlation between alcohol consumption and the levels of PEth in blood.



PEth has been shown to be a more sensitive indicator of alcohol consumption than other markers. The half-life of PEth in circulation is around 4 days, which means that PEth practically can be detected up to 4 weeks after ethanol has been cleared from the body.

In a blood test the PEth concentrations could be very accurately be quantify by LC-MS/MS analyze.

The test developed by redhot diagnostics is a robust system with high specificity and sensitivity.

### 3. PRINCIPLES OF THE PROCEDURE

Phosphatidylethanol is extracted from whole blood by addition of 200  $\mu$ L of extraction

buffer, containing an internal standard (deuterated phosphatidylethanol), to 50 µL of blood. After thorough mixing the tube is centrifuged and an aliquot of the supernatant is injected in the LC-MS system. The components are separated on a reversed phase (C8) column using a binary gradient. The effluent from column is monitored with electrospray ionization mass spectrometry using multiple reaction monitoring (MRM) to follow the respective characteristic transitions for PEth and the internal standard. The ratios of the chromatographic peak areas for PEth to the internal standard are used to quantify the concentration of PEth in the samples.

#### 4. WARNING AND PRECAUTIONS

Materials included in this kit should not be used past the expiration date on the kit label.

Reagents or substrates included in this kit should not be mixed or substituted with reagents or substrates from other kits.

Variation in pipetting, washing, incubation time or temperature may cause differences for the results.

The assay is designed to eliminate interference and background by other cellular macromolecules or factors present within any blood samples. The possibility of background noise cannot be fully excluded until all factors have been tested using the assay kit.

#### 5. HEALTH AND SAFETY PRECAUTIONS

Please wear proper eye, hand and face protection when handling this material. When the experiment is finished, be sure to discard residues in accordance with regulations

#### 6. KIT CONTENTS

##### Art. no. 50-2002

Label	Kit Component	Quantity
CAL	PEth 16:0/18:1	40 nmol/0,5 ml
INT. STD	D5-PEth 16:0/18:1	2X16 nmol/0,5 ml
EXT	Extraction solution	2x40 ml
TUN	Tuning solution	1 ml

As a first step for the application of the PEth LC-MS/MS-Kit, a tuning is necessary to estimate the optimal LC-MS/MS-settings as well as to assess the sufficiency of the sensitivity.

#### 7. STORAGE CONDITIONS

The reagents should be stored at +2-8°C

The Calibrator when diluted in whole blood should be stored frozen at -20°C

## 8. MATERIALS REQUIRED BUT NOT SUPPLIED

- LC-MS/MS-Equipment
- Column
- Mobile phase A
- Mobile phase B
- Vortex-Mixer
- Centrifuge
- Pre-columns or filter
- Vials for sample extraction procedure
- Autosampler vials (glass)

Recommended column Raptor FluoroPhenyl C8 column:

**Description:** Kinetex® 2.6 µm C8 100 Å, LC Column 50 x 2.1 mm, Ea

**Stationary Phase:** C8 with TMS end capping

**Solid Support:** Core-shell Silica

Separation Mode: Reversed Phase

## 9. PREPARATION OF REAGENTS

Whole blood samples (collected in EDTA vials) are suitable for the assay, either fresh or frozen blood samples can be used with the assay.

Prior to use allow samples and reagents to come to room temperature.

Important! Centrifuge the ampules containing calibrator and internal standard briefly before opening it to continue the procedure (e.g Eppendorf centrifuge, 2000 RCF, 2 min).

### Preparation of extraction solution (EXT)

Centrifuge the ampule containing internal standard D5-PEth 16:0/18:1 (2000 RCF, 2 min), open the ampule and add 0.5 ml Ext. sol., transfer the solution to the Ext. sol. bottle, repeat 2 times to quantitatively transfer the internal standard to the Ext. sol. bottle. The final concentration of d5- PEth 16:0/18:1 internal standard in Ext. sol. is 0.4 µM.

### Calibrator Preparation and Dilution

Pre-screen blank blood to assure it is free from PEth or other interferences. Centrifuge the ampule containing Calibrator PEth 16:0/18:1 (2000 RCF, 2 min), open the ampule and add 500 µL blood to the PEth 16:0/18:1 calibrator ampule. Mix thoroughly with the pipette. Use this as stock solution when preparing the C1 calibrator and the C1 to prepare the C2 etc. according to the table and figure below. The resulting vials C1-C5 can be used to prepare at least eight calibration curves when taking 50 microliter blood from each vial to prepare the calibration curve. The calibrator curve should be stored at -20°C

Standard	Standard concentration ( $\mu\text{M}$ )	Stock	Vol. Stock [ $\mu\text{l}$ ]	Vol. diluent [ $\mu\text{l}$ ]	Total vol. [ $\mu\text{l}$ ]	Final vol. [ $\mu\text{l}$ ]
Cal	40				100	900
C1	4	C1	100	900	100	750
C2	1	C2	250	750	100	500
C3	0.4	C3	500	500	1000	800
C4	0.1	C4	200	800	100	500
C5	0.05	C5	500	500	1000	1000

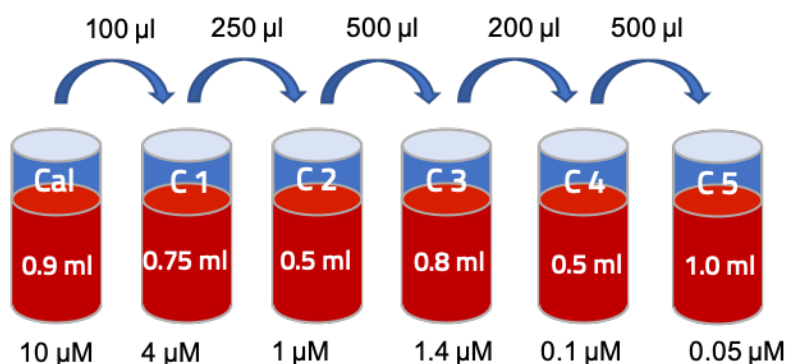


Figure 1. Serial dilution of calibrator in whole blood

### Mobile phase A

Production volume 750 ml

Substance, final conc.	Volume
20% acetonitrile	150 mL
0,5 mM ammonia	375 $\mu\text{L}$
1,0 mM acetic acid	750 $\mu\text{L}$
dH <sub>2</sub> O	600 mL

### Mobile phase B

Production volume 500 ml

Substance, final conc.	Volume
20% acetonitrile	100 mL
20% tetrahydrofuran	100 mL
60% 2-propanol	300 mL

## 10.SAMPLE PREPARATION

1. To 50  $\mu\text{L}$  blood add 200  $\mu\text{L}$  Ext sol., vortex thoroughly for 2 x 5 sec.
2. Centrifuge the tubes at 16400 RCF at room temperature for 10 min.

- Transfer 200  $\mu$ L of the supernatant to an autosampler vial, place the vial in the autosampler inject 20  $\mu$ L of the sample into the instrument

### 11. CHROMATOGRAPHIC CONDITIONS LC-MS/MS METHOD (LISTED AS AN EXAMPLE)

Instrument	Waters Quattro Premier XE
Ionization	Electrospray
Scan Type	MRM
Polarity	Negative
Spray Voltage (kV)	2.8
Cone Gas (L/hrs.)	100
Desolvation Temp ( $^{\circ}$ C)	370
Source Temp ( $^{\circ}$ C)	90
Desolvation Gas Flow (L/hrs.)	800
Collision Gas (mL/min)	0.1
Cone Voltage (V)	60

### 12. GRADIENT

Mobile phase A and Mobile Phase B Flow is 0.3 mL/min

Time	MOP A	MOP B
0.00	70	30
1.5	20	80
1.6	0	100
2.2	0	100
2.25	70	30

### 13. CALCULATION

The peak areas for the analytes are divided with the peak areas for the internal standard, and the ratios (responses) are used to fit straight lines to the response vs the concentration of the analyte in the calibration samples. First order linear regression weighted by  $1/x$  is preferred.

## 14. EXAMPLES OF CHROMATOGRAMS

Molecule	MW
PEth 16:0/18:1	703
PEth-d5 16:0/18:1	708

TWO DIFFERENT MRM TRANSITION FRAGMENTS 255.6 AND 281.6.

It is recommended to use the  $m/z$  701  $\rightarrow$  255 MRM transition to record the chromatograms, and to use the same fragment (255) for the PEth internal standard. The PEth tuning solution will simplify finding the exact decimals in the  $m/z$  values for each MS/MS instrument.

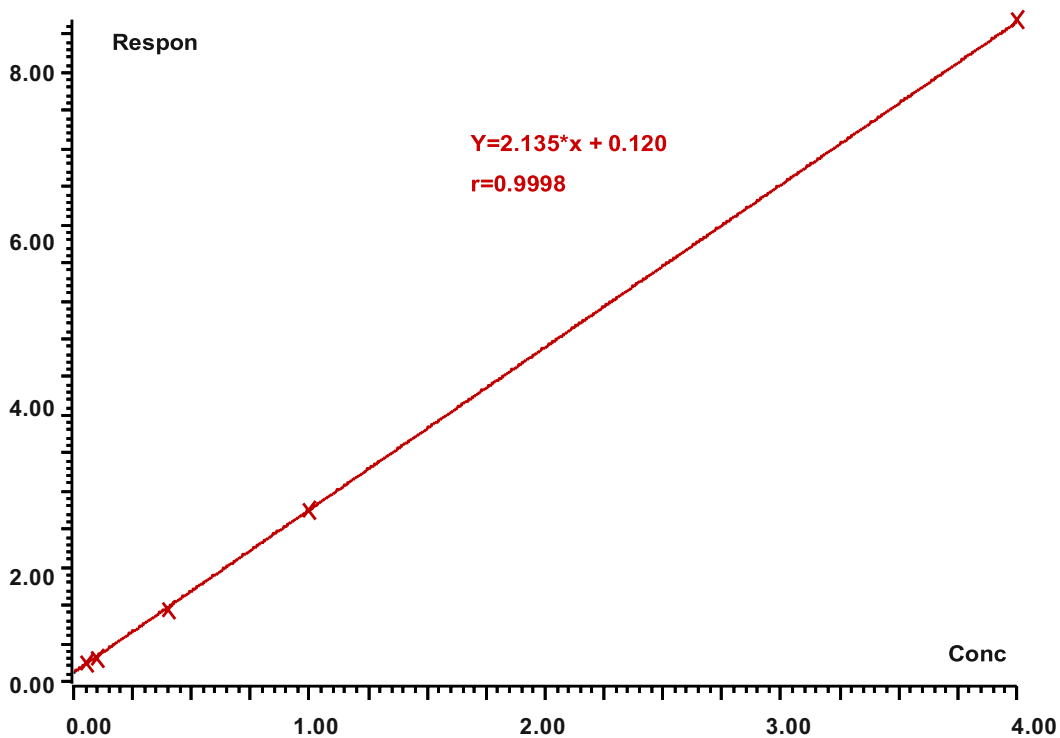


Figure 2. Calibration curve based on chromatograms from the 701  $\rightarrow$  255 transition



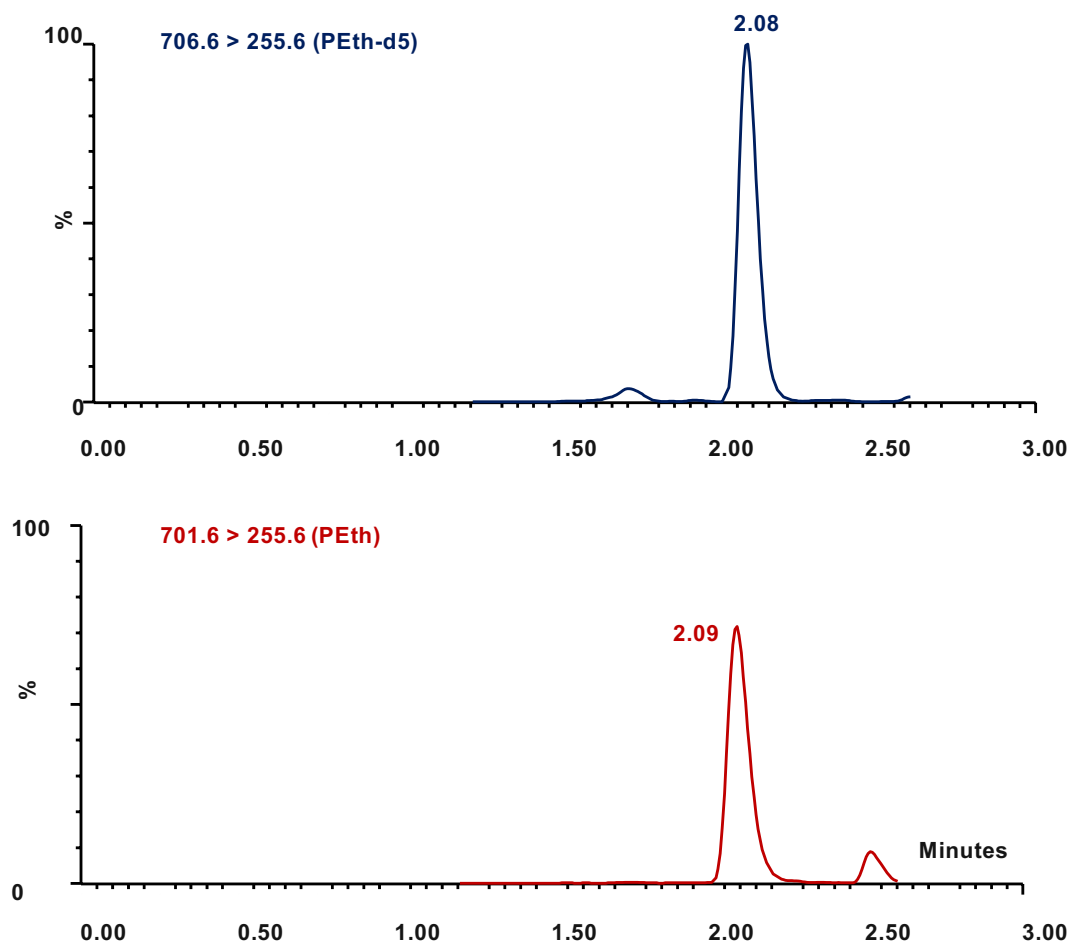


Figure 3. Control sample at 0.3  $\mu$ M

## 15.QUALITY CONTROL

Control samples should be analyzed with each batch of samples. Results generated from the analysis of control samples should be evaluated by statistical methods to ensure that the method shows accurate results. It is recommended to monitor the internal standard area for all batches. A systematic decrease of the internal standard area during several batches can indicate hardware related issues such as contaminated column or ion source. Individual outliers can indicate issues with the sample or the preparation of the sample.

### Set-up solution:

Use the included PEth tuning solution to find the exact transitions for the MRM traces when setting up the kit for the first time. Check the accuracy of the mass scales after annual preventive maintenance of the mass spectrometer, and after all other manipulations which can affect the accuracy of the mass scales.

### Wash solution:

Follow the recommendations from the manufacturer of the autosampler.

**Internal standard:**

The area for the internal standard should be consistent. If the area is inconsistent it is a sign of interference and/or carryover effects.

**16.PERFORMANCE CHARACTERISTICS****Detection level**

0.05 µM

**Measuring range**

0.05 - 4 µM

Samples over 4 µM should be diluted and analyzed again.

**Reproducibility**

Sample	PEth [µmol/L]	Intra Assay CV (n=20)	Inter Assay CV (n=20)
QC low	0.15	<12%	<15%
QC Mid	0.7	<10%	<12%
QC High	3.0	<8%	<10%

**17.CE MARKING**

The PEth kit is CE marked according to the EC in vitro diagnostic directive 98/79/EC.

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