Enzymatic Assay of PHOSPHOLIPASE C
(EC 3.1.4.3)
from Bacillus cereus

PRINCIPLE:

Lecithin + H$_2$O $\xrightarrow{\text{Phospholipase C}}$ Diglyceride + Choline Phosphate

Choline Phosphate + H$_2$O $\xrightarrow{\text{Alkaline Phosphatase}}$ Choline + P$_i$

Choline + O$_2$ $\xrightarrow{\text{Choline Oxidase}}$ Betaine Aldehyde + H$_2$O$_2$

Betaine Aldehyde + O$_2$ $\xrightarrow{\text{Choline Oxidase}}$ Betaine + H$_2$O$_2$

2 H$_2$O$_2$ + Phenol + 4-Aminoantipyrine $\xrightarrow{\text{Peroxidase}}$ 4 H$_2$O + Quinoneimine Dye

Abbreviations used:
Lecithin = L-$\alpha$-Phosphatidylcholine
P$_i$ = Inorganic Phosphate

CONDITIONS: T = 37°C, pH = 7.3, A$_{500\text{nm}}$, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

A. 100 mM Dimethylglutaric Acid Buffer, pH 7.3 at 37°C
   (Prepare 100 ml in deionized water using 3,3-Dimethylglutaric Acid, Sigma Prod.
   No. D-4379. Adjust to pH 7.3 at 37°C with 2 M NaOH.)

B. 5.0% (v/v) Triton$^2$ X-100 Solution
   (Prepare 25 ml in deionized water using Triton X-100, Sigma Stock No. X-100.)

C. 4.0% (w/v) Lecithin Substrate Solution (Lecithin)
   (Prepare 25 ml in Reagent B using L-$\alpha$-Phosphatidylcholine, Sigma Prod. No. P-5638.
   PREPARE FRESH.)

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REAGENTS: (continued)

D.  1 M Tris Buffer with 0.2% (w/v) Sodium Dodecyl Sulfate, pH 8.0 at 37°C (Tris-SDS)  
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503, and Lauryl Sulfate, Sodium Salt, Sigma Prod. No. L-4509. Adjust to pH 8.0 at 37°C using 5 M HCl.)

E.  50 mM Tris Buffer, pH 8.0 at 37°C (Tris Buffer)  
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 8.0 at 37°C using 1 M HCl.)

F.  148 mM 4-Aminoantipyrine Solution (4-AAP)  
(Prepare 1 ml in deionized water using 4-Aminoantipyrine, Sigma Prod. No. A-4382.)

G.  2.0% (w/v) Phenol Solution (Phenol)  
(Prepare 1 ml in deionized water using Phenol, Sigma Prod. No. P-4161.)

H.  Choline Oxidase Enzyme Solution (Choline Oxidase)  
(Immediately before use, prepare a solution containing 50 units/ml in Reagent E using Choline Oxidase, Sigma Prod. No. C-5896.)

I.  Peroxidase Enzyme Solution (Peroxidase)  
(Immediately before use, prepare a solution containing 2500 units/ml in deionized water using Peroxidase, Sigma Prod. No. P-8250.)

J.  Alkaline Phosphatase Enzyme Solution (Alkaline Phosphatase)  
(Immediately before use, prepare a solution containing 1440 units/ml in deionized water using Phosphatase, Alkaline, Sigma Prod. No. P-5521.)

K.  10 mM Dimethylglutaric Acid Buffer with 0.1% (w/v) Bovine Serum Albumin, pH 7.3 at 37°C  
(Enzyme Diluent)  
(Prepare 100 ml in deionized water using 3,3-Dimethylglutaric Acid, Sigma Prod. No. D-4379, and Albumin, Bovine, Sigma Prod. No. A-4503. Adjust to pH 7.3 at 37°C with 2 M NaOH.)

L.  Phospholipase Enzyme Solution (Phospholipase)  
(Immediately before use, prepare a solution containing 0.08 - 0.2 unit/ml in cold Reagent K.)
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PROCEDURE:

Prepare the Chromogen Solution by pipetting (in milliliters) the following reagents into a suitable container:

- Reagent E (Tris Buffer) 4.50
- Reagent F (4-AAP) 0.05
- Reagent G (Phenol) 0.05
- Reagent H (Choline Oxidase) 0.30
- Reagent I (Peroxidase) 0.02
- Deionized Water 0.08

Mix by swirling.

Pipette (in milliliters) the following reagents into suitable containers:

Reaction Cocktail

- Deionized water 2.00
- Reagent A (Buffer) 4.00
- Reagent C (Lecithin) 3.00

Mix by inversion and adjust pH to 7.3 at 37°C with either 1 N HCL or 1 N NaOH.

Pipette (in milliliters) the following reagents into a suitable vial:

<table>
<thead>
<tr>
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<th>Test</th>
<th>Blank</th>
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<tbody>
<tr>
<td>Reaction Cocktail</td>
<td>0.90</td>
<td>0.90</td>
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Mix by inversion and equilibrate to 37°C. Then add:

- Reagent L (Phospholipase C) 0.05

Mix by inversion and incubate at 37°C for exactly 10 minutes. Then add:

- Reagent D (Tris-SDS) 1.00
- Reagent L (Phospholipase C) 0.05

Mix by inversion. Then add:

- Chromogen Solution 1.00
- Reagent J (Alkaline Phosphatase) 0.01
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PROCEDURE:  (continued)

Mix by inversion and incubate at 37°C for 20 minutes. Cool to 25°C and filter through a AP-20 borosilicate glass fiber, pore sizes: 0.45 µm membrane and a Cellulose Acetate, pore size: 0.20 µm membrane combination, Sigma Prod. No. F8773 and F0139, respectively. Transfer solutions to suitable cuvettes and read the $A_{500\text{nm}}$ for the Test and Blank.

CALCULATIONS:

$$\text{Units/ml enzyme} = \frac{(A_{500\text{nm Test}} - A_{500\text{nm Blank}})(2.96)(df)}{(12)(10)(0.05)}$$

2.96 = Total volume (in milliliters) of assay  
df = Dilution factor  
12 = Millimolar extinction coefficient$^4$ of quinoneimine dye at 500 nm  
10 = Time (in minutes) of the assay as per the Unit Definition  
0.05 = Volume (in milliliter) of enzyme used

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}}$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}}$$

UNIT DEFINITION:

One unit will liberate 1.0 micromole of water soluble organic phosphorus from L-α-phosphatidylcholine per minute at pH 7.3 at 37°C.

FINAL ASSAY CONCENTRATION:

In a 0.95 ml reaction mix, the final concentrations are 42 mM dimethylglutaric acid, 1.6% (v/v) Triton X-100, 0.63% (w/v) lecithin, and 0.004 - 0.01 unit phospholipase C.
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REFERENCES:

Biochemicals, Indianapolis, IN

Toyo Jozo Co, Ltd. (1982) in Toyo Jozo Enzymes, pp. 21-22, Tokyo, Japan

NOTES:

1. This product is also assayed using L-α-Phosphatidylcholine Type IX-E, from Fresh Frozen Egg
   Yolk, Sigma Prod. No. P-9671

2. Triton is a registered trademark of Union Carbide.

3. Lecithin requires ample time in "solution" in order to obtain a homogenous suspension. This
   concentrations represents 4.0 g of P5638 in 100 ml of Reagent B. There is no correction for
   acetate lecithin content.

4. The millimolar extinction coefficient of quinoneimine dye is described in Keesey, J. (1987).

5. Alkaline Phosphatase Unit Definition: One unit will hydrolyze 1.0 µmole of p-nitrophenyl
   phosphate per minute at 37°C.

6. Choline Oxidase Unit Definition: One unit will form 1.0 µmole of H₂O₂ from choline and H₂O per
   minute at pH 8.0 at 37°C.

7. Peroxidase Unit Definition: One unit will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0
   at 20°C.

8. This assay is based on the cited references.

9. Where Sigma Product or Stock numbers are specified, equivalent reagents may be
   substituted.

This procedure is for informational purposes. For a current copy of Sigma’s quality control
procedure contact our Technical Service Department.