Enzymatic Assay of PHOSPHOLIPASE C
(EC 3.1.4.3)

PRINCIPLE:

Lecithin + H_2O $\xrightarrow{\text{Phospholipase C}}$ Diglyceride + Choline Phosphate

Choline Phosphate + H_2O $\xrightarrow{\text{Alkaline Phosphatase}}$ Choline + P_i

Choline + O_2 $\xrightarrow{\text{Choline Oxidase}}$ Betaine Aldehyde + H_2O_2

Betaine Aldehyde + O_2 $\xrightarrow{\text{Choline Oxidase}}$ Betaine + H_2O_2

2 H_2O_2 + Phenol + 4-Aminoantipyrine $\xrightarrow{\text{Peroxidase}}$ 4 H_2O + Red Dye

Abbreviations:
Lecithin = L-a-Phosphatidylcholine
P_i = Inorganic Phosphate

CONDITIONS: T = 37°C, pH = 7.3, A_{500nm}, 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 X-100[1] Solution
(Prepare 25 ml in deionized water using Triton X-100, Sigma Stock No. X-100.)

C. 26 mM Lecithin Substrate Solution (Lecithin)
(Prepare 25 ml in Reagent B using L-a-Phosphatidylcholine, Sigma Prod. No. P-9671.)

D. 1 M Tris Buffer with 0.2% (v/v) SDS, 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.)

REAGENTS: (continued)

E. 50 mM Tris Buffer, pH 8.0 at 37°C
(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. 150 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. 11 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 or equivalent. Adjust to pH 7.3 at 37°C with 2 M NaOH.)

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

Enzymatic Assay of PHOSPHOLIPASE C (EC 3.1.4.3)

PROCEDURE:

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

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 the following reagents into suitable vials (in milliliter):

	Test	Blank
Deionized Water	0.20	0.20
Reagent A (Buffer)	0.40	0.40
Reagent C (Lecithin)	0.30	0.30

Mix by inversion and equilibrate to 37°C. Then add:

Reagent L (Phospholipase)	0.05	------

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

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

Mix by inversion.

COLORIMETRIC ASSAY:

Add the following reagents:

Chromogen Solution	1.00	1.00
Reagent J (Alkaline Phosphatase)	0.01	0.01

Mix by inversion and incubate at 37°C for 20 minutes. Cool to 25°C and transfer the solutions to suitable cuvettes. Read the A_{500nm} for the Test and Blank.

Enzymatic Assay of PHOSPHOLIPASE C
(EC 3.1.4.3)

CALCULATIONS:

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

2.96 = Total volume (in milliliters) of assay
df = Dilution factor
12 = Millimolar Extinction Coefficient of the Red dye
10 = Time of assay in (minutes) 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-a-phosphatidylcholine per minute at pH 7.3 at 37°C.

FINAL ASSAY CONCENTRATION:

In a 0.95 ml reaction mix, the final concentrations are 43 mM dimethylglutaric acid, 0.005% (w/v) bovine serum albumin, 8.2 mM lecthin, 1.6% (v/v) Triton X-100 and 0.01 unit phospholipase C.

NOTES:

1. Triton is a registered trademark of Union Carbide.

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

3. Choline Oxidase Unit Definition: One unit will form 1.0 µmole of H_2O_2 from choline and H_2O per minute at pH 8.0 at 37°C.

NOTES: (continued)

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

5. All product and stock numbers, unless otherwise indicated, are Sigma product and stock numbers.

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