

**Enzymatic Assay of PHOSPHOLIPASE C  
(EC 3.1.4.3)  
from *C. perfringens***

**PRINCIPLE:**

L- $\alpha$ -Lecithin + H<sub>2</sub>O  $\xrightarrow{\text{Phospholipase C}}$  1,2-Diglyceride + Choline Phosphate

Choline phosphate + H<sub>2</sub>O  $\xrightarrow{\text{Alkaline Phosphatase}}$  Choline + P<sub>i</sub>

Abbreviations used:

L- $\alpha$ -Lecithin = L- $\alpha$ -Phosphatidylcholine

P<sub>i</sub> = Inorganic Phosphate

**CONDITIONS:** T = 37°C, pH 7.3, A<sub>660nm</sub>, Light path = 1 cm

**METHOD:** Colorimetric

**REAGENTS:**

- A. 50 mM Tris Maleate Buffer, pH 7.3 at 37°C  
(Prepare 100 ml in deionized water using Trizma Maleate, Sigma Prod. No. T-3128. Adjust to pH 7.3 at 37°C with 10 M NaOH.)
- B. 50 mM Calcium Chloride Solution (CaCl<sub>2</sub>)  
(Prepare 25 ml in Reagent A using Calcium Chloride, Dihydrate, Sigma Prod. No. C-3881.)
- C. 2.0% (w/v) L- $\alpha$ -Phosphatidylcholine (Lecithin)  
(Prepare 25 ml in deionized water using L- $\alpha$ -Phosphatidylcholine, from Fresh Frozen Egg Yolk, Sigma Prod. No. P-9671.)
- D. 50 mM Tris Maleate Buffer with 1.0% (w/v) Bovine Serum Albumin, pH 7.3 at 37°C  
(Enzyme Dil)  
(Prepare 25 ml in Reagent A using Albumin, Bovine, Sigma Prod. No. A-4503.)

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**REAGENTS:** (continued)

- E. 270 mM Ethylenediaminetetraacetic Acid Solution, pH 7.3 at 37°C (EDTA)  
(Prepare 25 ml in deionized water using Ethylenediaminetetraacetic Acid, Tetrasodium Salt, Hydrate, Sigma Prod. No. ED4S. Adjust to pH 7.3 at 37°C with 5 M HCl.)
- F. Alkaline Phosphatase Enzyme Solution (Alk Phos)  
(Immediately before use, prepare a solution containing 40 units/ml in deionized water using Phosphatase, Alkaline, Sigma Prod. No. P-4377.)
- G. 20% (w/v) Lauryl Sulfate Solution (SDS)  
(Prepare 25 ml in deionized water using Lauryl Sulfate, Sodium Salt, Sigma Prod. No. L-4509.)
- H. Phosphorus Std (P Std)  
(Use Phosphorus Standard Solution, Sigma Stock No. 661-9. The Phosphorus concentration is 20 µg/ml, 0.645 µ/mole.)
- I. 10% (w/v) Ascorbic Acid Solution (Ascorbic Acid)  
(Prepare 25 ml in deionized water using L-Ascorbic Acid, Sodium Salt, Sigma Prod. No. A-7631.)
- J. 4.2% (w/v) Molybdic Acid Solution (Molyb Acid)  
(Prepare 25 ml in 10 N H<sub>2</sub>SO<sub>4</sub> using Molybdic Acid, Ammonium Salt, Tetrahydrate, Sigma Prod. No. M-0878 and Sulfuric Acid, Sigma Prod. No. S-1526.)
- K. Ames Color Reagent (Clr Rgt)  
(Prepare by combining 10 ml of Reagent I (Ascorbic Acid), 6 ml Reagent J (Molyb Acid) and 54 ml of deionized water. Mix by swirling and store in the dark at room temperature. This reagent should be prepared 30 minutes before use.)
- L. Phospholipase C Enzyme Solution (Phospholipase C)  
(Immediately before use, prepare a solution containing 0.1-1.0 unit/ml in cold Reagent D.)

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**PROCEDURE:**

Pipette (in milliliters) the following reagents into 4 dram vials:

	Test	Test Blank	Std 1	Std 2	Std 3	Std 4	Std Blank
Reagent A (Buffer)	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Reagent B (CaCl <sub>2</sub> )	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Reagent C (Lecithin)	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Reagent D (Enzyme Dil)	0.90	0.90	--	--	--	--	--
Reagent H (P Std)	--	--	0.25	0.50	0.75	1.00	--
Deionized Water	--	--	0.75	0.50	0.25	--	1.00

Mix by swirling and equilibrate at 37°C. Then add:

Reagent L (Phospholipase C)	0.10	--	--	--	--	--	--
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Mix by swirling and incubate at 37°C for exactly 15 minutes. Then add:

Reagent E (EDTA)	0.90	0.90	0.90	0.90	0.90	0.90	0.90
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Mix by swirling. Then add:

Reagent L (Phospholipase C)	--	0.10	--	--	--	--	--
Reagent F (Alk Phos)	0.10	0.10	0.10	0.10	0.10	0.10	0.10

Mix by swirling and incubate at 37°C for 120 minutes.

Pipette (in milliliters) the following reagents into 4 dram vials:

Test Solution	1.00	--	--	--	--	--	--
Test Blank Solution	--	1.00	--	--	--	--	--
Standard 1	--	--	1.00	--	--	--	--
Standard 2	--	--	--	1.00	--	--	--
Standard 3	--	--	--	--	1.00	--	--
Standard 4	--	--	--	--	--	1.00	--
Standard Blank	--	--	--	--	--	--	1.00

Mix by swirling. Then add:

Reagent G (SDS)	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Reagent K (Clr Rgt)	3.00	3.00	3.00	3.00	3.00	3.00	3.00

Mix by swirling and incubate at 37°C for 60 minutes. Centrifuge and transfer the Test, Test Blank, Standards, and Standard Blank to suitable cuvettes. Read the A<sub>660nm</sub> for each of the samples and blanks using a suitable spectrophotometer.

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**CALCULATIONS:**

Standard Curve:

$$\Delta A_{660\text{nm}} \text{ Standard} = A_{660\text{nm}} \text{ Standard} - A_{660\text{nm}} \text{ Standard Blank}$$

Prepare a Standard curve by plotting the  $\Delta A_{660\text{nm}}$  Standard versus micromoles of phosphorus.

Sample Determination:

$$\Delta A_{660\text{nm}} \text{ Test} = A_{660\text{nm}} \text{ Test} - A_{660\text{nm}} \text{ Test Blank}$$

Determine the micromoles of Phosphorus liberated using the Standard curve.

$$\text{Units/ml enzyme} = \frac{(\mu\text{moles of phosphate released})(df)}{(15)(0.1)}$$

df = Dilution factor

15 = Time of reaction (in minutes) as per the Unit Definition

0.1 = 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  $\mu\text{mole}$  of water soluble organic phosphorus from L- $\alpha$ -phosphatidylcholine per min at pH 7.3 at 37°C.

**FINAL ASSAY CONCENTRATION:**

In a 5.00 ml reaction mix, the final concentrations are 35 mM Tris maleate, 5 mM calcium chloride, 0.6% (w/v) L- $\alpha$ -phosphatidylcholine, 0.2% (w/v) bovine serum albumin, and 0.01 - 0.10 unit phospholipase c.

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**NOTES:**

1. Break up any large lumps of L- $\alpha$ -phosphatidylcholine and dissolve in deionized water. Sonicate at 30 second intervals for approximately 5 minutes or until the product is completely dissolved. The resulting suspension should be homogeneous.
2. Alkaline Phosphatase Unit Definition: One unit will hydrolyze 1.0  $\mu$ mole of p-nitrophenyl phosphate per minute at pH 9.8 at 37°C.
3. 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.**