SIGMA QUALITY CONTROL TEST PROCEDURE

**ProductInformation**

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

**PRINCIPLE:**

L-α-Lecithin + $H_2O$ $\xrightarrow{\text{Phospholipase C}}$ 1,2-Diglyceride + Choline Phosphate

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

Abbreviations used:
L-α-Lecithin = L-α-Phosphatidylcholine
$P_i$ = Inorganic Phosphate

**CONDITIONS:** T = 37°C, pH 7.3, $A_{660nm}$, 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 ($CaCl_2$)
(Prepare 25 ml in Reagent A using Calcium Chloride, Dihydrate, Sigma Prod. No. C-3881.)

C. 2.0% (w/v) L-α-Phosphatidylcholine (Lecithin)
(Prepare 25 ml in deionized water using L-α-Phosphatidylcholine, from Fresh Frozen Egg Yolk, Sigma Prod. No. P-9671.[1])

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.)

**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 $H_2SO_4$ 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 ($CaCl_2$) | 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 | --- | --- | --- | --- | --- | --- |

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 |

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 $A_{660nm}$ for each of the samples and blanks using a suitable spectrophotometer.

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**CALCULATIONS:**

Standard Curve:

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

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

Sample Determination:

$\Delta A_{660nm}$ Test = $A_{660nm}$ Test - $A_{660nm}$ 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 μmole of water soluble organic phosphorus from L-α-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-α-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-α-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 µmole 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.