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

PRINCIPLE:

\[ \text{L-}\alpha\text{-Lecithin} + \text{H}_2\text{O} \xrightarrow{\text{Phospholipase C}} \text{1,2-Diglyceride} + \text{Choline Phosphate} \]

\[ \text{Choline phosphate} + \text{H}_2\text{O} \xrightarrow{\text{Alkaline Phosphatase}} \text{Choline} + \text{P}_i \]

**Abbreviations used:**

- L-\alpha-Lecithin = L-\alpha-Phosphatidylcholine
- P\textsubscript{i} = Inorganic Phosphate

**CONDITIONS:** \( T = 37^\circ\text{C}, \text{pH} 7.3, A_{660\text{nm}}, \text{Light path} = 1 \text{ cm} \)

**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\textsubscript{2})
   (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-9671.1)

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.)
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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)

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:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Test</th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reagent A (Buffer)</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
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<tr>
<td>Reagent B (CaCl₂)</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Reagent C (Lecithin)</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
<td>1.50</td>
</tr>
<tr>
<td>Reagent D (Enzyme Dil)</td>
<td>0.90</td>
<td>0.90</td>
<td>---</td>
<td>---</td>
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<td>---</td>
</tr>
<tr>
<td>Reagent H (P Std)</td>
<td>---</td>
<td>---</td>
<td>0.25</td>
<td>0.50</td>
<td>0.75</td>
<td>1.00</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>---</td>
<td>---</td>
<td>0.75</td>
<td>0.50</td>
<td>0.25</td>
<td>1.00</td>
<td>---</td>
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</tr>
</tbody>
</table>

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 | 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 | 0.10 |

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

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

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Test</th>
<th>Std 1</th>
<th>Std 2</th>
<th>Std 3</th>
<th>Std 4</th>
<th>Std</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test Solution</td>
<td>1.00</td>
<td>---</td>
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<td>---</td>
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<td>---</td>
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</tr>
<tr>
<td>Test Blank Solution</td>
<td>---</td>
<td>1.00</td>
<td>---</td>
<td>---</td>
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<td>---</td>
</tr>
<tr>
<td>Standard 1</td>
<td>---</td>
<td>---</td>
<td>1.00</td>
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</tr>
<tr>
<td>Standard 2</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>1.00</td>
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<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Standard 3</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>1.00</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Standard 4</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>1.00</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Standard Blank</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>1.00</td>
<td>---</td>
</tr>
</tbody>
</table>

Mix by swirling. Then add:

Reagent G (SDS) | 0.50 | 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 | 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 A₆₆₀nm for each of the samples and blanks using a suitable spectrophotometer.
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**CALCULATIONS:**

Standard Curve:

\[
\Delta A_{660} = A_{660} \text{ Standard} - A_{660} \text{ Standard Blank}
\]

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

Sample Determination:

\[
\Delta A_{660} = A_{660} \text{ Test} - A_{660} \text{ Test Blank}
\]

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 \( \mu \)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-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.

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