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ProductInformation

SIGMA QUALITY CONTROL TEST PROCEDURE

Enzymatic Assay of PHOSPHOLIPASE D[1]
(EC 3.1.4.4)

PRINCIPLE:

L-α-Phosphatidylcholine + $2H_2O$ $\xrightarrow{\text{Phospholipase D}}$ Choline + Phosphatidic Acid

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

$2H_2O_2$ + 4-AAP + Phenol $\xrightarrow{\text{Peroxidase}}$ $4H_2O$ + Quinoneimine Dye

Abbreviation used:
4-AAP = 4-Aminoantipyrine

CONDITIONS: T = 30°C, pH = 5.6, A_{500nm}, Light path = 1 cm

METHOD: Colorimetric

REAGENTS:

A. 50 mM Sodium Lauryl Sulfate Solution (SDS)
(Prepare 10 ml in deionized water using Lauryl Sulfate, Sodium Salt, Sigma Prod. No. L-5750.)

B. 1 M Sodium Acetate Buffer, pH 5.6 at 30°C (NaOAc)
(Prepare 100 ml in deionized water using Sodium Acetate, Trihydrate, Sigma Prod. No. S-8625. Adjust to pH 5.6 at 30°C with 1 M HCl.)

C. 17.9% (v/v) Ethanol Solution (EtOH)
(Prepare 1 ml in deionized water using 200 Proof USP Ethyl Alcohol, available from Quantum Chemical Company.)

D. 0.46% (w/v) L-α-Phosphatidylcholine Substrate Solution
(Prepare by transferring 2.2 ml (220 mg) of L-α-Phosphatidylcholine, Sigma Prod. No. P-5388, to a 50 ml Erlenmeyer flask. Evaporate off the hexane by bubbling nitrogen gas through the liquid. Place the Erlenmeyer flask containing the substrate into a desiccator connected to a vacuum line for 4 hours. Add in order: 3 ml of Reagent A (SDS), 6 ml of Reagent B (Buffer), and 39 ml of deionized water. Mix, using a magnetic stirrer, until a uniform suspension is obtained. Add 0.272 ml of Reagent C (EtOH) to obtain a 0.1% (v/v) ethanol concentration in the substrate solution. **PREPARE FRESH**.)

E. 500 mM Calcium Chloride Solution ($CaCl_2$)
(Prepare 25 ml in deionized water using Calcium Chloride, Dihydrate, Sigma Prod. No. C-3881.)

F. 100 mM Tris HCl Buffer, pH 8.0 at 30°C
(Prepare 100 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 8.0 at

30◦C with 1 M HCl.)

G. 10 mM Tris HCl Buffer with
2 mM Ethylenediaminetetraacetic Acid and 1.0% (w/v) Potassium Chloride (Enzyme Diluent)
(Prepare 10 ml in Reagent F using Ethylenediaminetetraacetic Acid, Disodium Salt, Dihydrate, Sigma Stock No. ED2SS and Potassium Chloride,
Sigma Prod. No. P-4504.)

H. Choline Oxidase Enzyme Solution (COD)
(Prepare a solution containing 10 units/ml of Choline Oxidase, Sigma Prod. No. C-5896, in cold Reagent G.)

I. 1 mM Choline Chloride Standard (Chol Std Soln)
(Prepare 50 ml in deionized water using Choline Chloride Salt, Sigma Prod. No. C-1879. **PREPARE FRESH**.)

J. Choline Color Reagent Mixture (CCRM)
(Prepare by dissolving 39 mg of 4-Aminoantipyrine, Free Base, Sigma Prod. No. A-4382, 80 mg of Phenol, Sigma Prod. No. P-3653 and 8 mg of Peroxidase, Sigma Prod. No. P-8250 in 5.5 ml of Reagent F (Tris Buffer, pH 8.0). Store in an amber bottle to protect from light.)

K. 2 M Tris HCl Buffer, pH 9.0 at 25°C
(Prepare 25 ml in deionized water using Trizma Base, Sigma Prod. No. T-1503. Adjust to pH 9.0 at 25°C with 1 M HCl.)

L. Phospholipase D Enzyme Solution (PLD)
(Immediately before use, prepare a solution containing 10 - 20 units/ml in cold deionized water.)

PROCEDURE:

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

	Test	Blank
Reagent D (Substrate Soln)	2.40	2.40
Reagent E ($CaCl_2$)	0.30	0.30
Deionized Water	0.20	0.30

Mix by swirling and equilibrate to 30°C using a thermostatted water bath. Then add:

Reagent L (PLD)	0.10	------

Immediately mix by swirling and incubate the containers for exactly 10 minutes at 30°C. The containers should be swirled several times during the reaction. At the end of 10 minutes, transfer the Test and Blank to a boiling water bath. Remove tubes from the water bath after 5 minutes and let cool to room temperature. Add 0.05 ml of Reagent K (Tris HCl Buffer). Mix, centrifuge and filter both Test and Blank through a 0.45 μm filter. Pipette (in milliliters) the following reagents into suitable containers.

Test Filtrate	2.00	------
Blank filtrate	------	2.00
Reagent J (CCRM)	0.10	0.10
Reagent H (COD)	0.10	0.10

Mix by inversion and let stand 2-3 hours at room temperature. Then add:

Deionized Water	2.00	2.00

Centrifuge to clarify and then transfer the solutions to suitable cuvettes. Record the A_{500nm} for both Test and Blank using a suitable spectrophotometer.

COLORIMETRIC ASSAY:

Standard Curve:

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

	Std 1	Std 2	Std 3	Std 4	Std Blank
Reagent D (Substrate Soln)	2.40	2.40	2.40	2.40	2.40
Reagent E ($CaCl_2$)	0.30	0.30	0.30	0.30	0.30
Reagent I (Chol Std Soln)	0.05	0.10	0.20	0.30	----
Deionized Water	0.25	0.20	0.10	----	0.30

Mix vigorously by vortexing and then place Standard and Standard Blank in a boiling water bath. Remove tubes after 5 minutes from the water bath and let cool to room temperature. Add 0.05 ml of Reagent K (Tris HCl Buffer), centrifuge and filter the Standards and Standard Blank through a 0.45 μm filter.

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

Std 1 Filtrate	2.00	----	----	----	----
Std 2 Filtrate	----	2.00	----	----	----
Std 3 Filtrate	----	----	2.00	----	----
Std 4 Filtrate	----	----	----	2.00	----
Blank Filtrate	----	----	----	----	2.00
Reagent J (CCRM)	0.10	0.10	0.10	0.10	0.10
Reagent H (COD)	0.10	0.10	0.10	0.10	0.10

Mix by inversion and let stand 2 - 3 hours at room temperature. Then add:

Deionized Water	2.00	2.00	2.00	2.00	2.00

Clarify the solutions by centrifugation. Transfer the solutions to cuvettes and record the A_{500nm} for both Standards and Standard Blank using a suitable spectrophotometer.

CALCULATIONS:

Standard Curve:

ΔA_{500nm} Standard = A_{500nm} Standard - A_{500nm} Standard Blank

Prepare a standard curve by plotting ΔA_{500nm} Standard versus the micromoles of Choline.

Sample Determination:

ΔA_{500nm} Sample = A_{500nm} Test - A_{500nm} Blank

Determine the total micromoles of Choline liberated using the Standard curve.

$$\text{Units/ml enzyme} = \frac{(\text{micromoles choline liberated})(6)(df)}{(0.1)}$$

6 = Time conversion factor for one hour (as per the Unit Definition)
0.1 = Volume (in milliliter) of enzyme used
df = Dilution factor

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

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

UNIT DEFINITION:

One unit will liberate 1.0 μmole of choline from L-α-phosphatidylcholine (egg yolk) per hour at pH 5.6 at 30°C.

FINAL ASSAY CONCENTRATION:

In a 3.00 ml reaction mixture, the final concentrations are
0.37% (w/v) L-α-phosphatidylcholine, 0.08% (v/v) ethanol,
99 mM sodium acetate, 2 mM sodium lauryl sulfate,
50 mM calcium chloride, and 1 - 2 units phospholipase D.

REFERENCE:

Artiss, J.D., Draisey, T.F., Thibert, R.J., Zak, B. and Taylor, K.E. (1980) *Microchemical Journal* **25**, 153-168

NOTES:

1. This assay should not be used to assay Phospholipase D, Type VI, Sigma Prod. No. P-8023.

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

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

4. This assay is based on the cited reference.

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