

Phospholipase D (from *Streptomyces chromofuscus*)
Phosphatidylcholine phosphatidohydrolase
(EC 3.1.4.4)

CATALOG NO.: BML-SE301 **LOT NO.:**

PURITY: >90% by SDS-PAGE; Apparent MW~60 kDa.

SPECIFIC ACTIVITY: U/mg protein. One unit will release 1 μ mol of choline from L- α -phosphatidylcholine (5 mM) per hour at pH 8.0 and 37°C (see assay conditions on next page).

USES: a) Catalyzes hydrolysis of phosphatidylcholine to phosphatidic acid and choline. Also active on phosphatidylserine, phosphatidylethanolamine, phosphatidylglycerol, lysophosphatidylcholine and sphingomyelins. Rate with 5 mM sphingomyelin is ~20% of the rate with the same concentration of phosphatidylcholine. b) Transferase activity: Phosphatidylcholine + n-alcohol \rightarrow phosphatidylalcohol + choline. (Some groups report that the transferase activity is low for *Streptomyces chromofuscus* Phospholipase D relative to Phospholipase D isolated from other species^{4,5}. We recommend Phospholipase D from *Streptomyces sp.* (Cat. # BML-SE302) for this reaction) c) Treatment of cultured cells (e.g. quiescent fibroblasts) with exogenous PLD can have growth-factor like effects², including activation of MAP kinase, stimulation of DNA synthesis and accumulation of GTP-bound Ras⁴. These effects may be due to PLD's generation of lysophosphatidic acid from lysophosphatidylcholine^{4,5}.

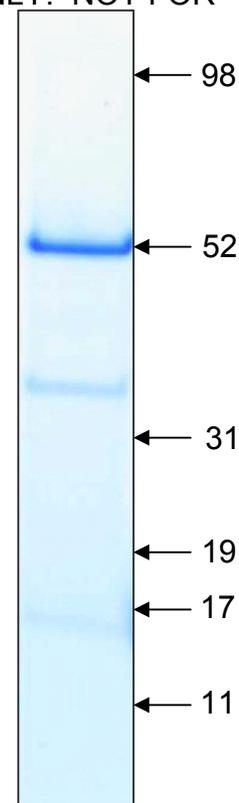
SUPPLIED AS: U/ml in 100 mM Tris/HCl, pH 8.0, 10% v/v glycerol, 0.1% w/v Triton X-100.

STORAGE: -70°C. After initial thaw, aliquot product into individual tubes, snap freeze and store at -70°C. Avoid repeated freeze/thaw cycles. NOTE: When stored under the above conditions, this enzyme is stable at the concentration supplied, in its current storage buffer. Procedures such as dilution of the enzyme followed by refreezing, could lead to loss of activity.

REFERENCES:

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5. T. Hagishita *et al. Anal. Biochem.* 1999 **276** 161
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5. P. Friedman *et al. J. Biol. Chem.* 1996 **271** 953

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DIAGNOSTIC OR
THERAPEUTIC USE.

2 μ g of phospholipase D [BML-SE301] resolved by SDS-PAGE (Novex, NuPage) and stained with Coomassie blue. Arrows and numbers indicate positions of marker proteins and their mass in kilodaltons.

Phospholipase D Assay

Catalog #BML-SE301

I. PRINCIPLES OF THE ASSAY

The assay comprises three reaction steps. In the first step, phospholipase D catalyzes hydrolysis of phosphatidylcholine to choline and phosphatidic acid. In the second step, oxidation of choline catalyzed by choline oxidase produces two hydrogen peroxides. Peroxidase catalyzes the third step, in which the two hydrogen peroxides, phenol and 4-aminoantipyrine react to produce a quinoneimine dye, with a millimolar extinction coefficient of $12.2 \text{ mM}^{-1}\text{cm}^{-1}$ at 500 nm. Measurement of the absorbance at 500 nm can then be used to calculate the amount of choline produced by the phospholipase reaction. Both the choline oxidase and peroxidase reactions take place in the same solution after addition of 'Reaction Mix II'. The addition of 'Reaction Mix II' also stops the phospholipase D reaction by addition of the chelator EDTA in excess of the calcium ion concentration. The procedure is written for use with a spectrophotometric plate reader and standard well 96-well microtiter plates. The final assay volume is 300 μl . With appropriate volume adjustments (1 or 3 ml final volume) reactions may also be read with standard or semi-micro cuvettes.

II. SOLUTIONS

Reaction Mix I (may be stored at -20°C)

44.4 mM Tris/HCl pH 8.0; 11.1 mM CaCl_2 ; 5.55 mM L--phosphatidylcholine, dioleoyl (C18:1, [cis]-9)/1.11% w/v Triton X-100 (added from a stock of 25 mM phosphatidylcholine in 5% Triton X-100)

Phospholipase D (prepare day of assay and keep on ice)

S. chromofuscus phospholipase D (BML-SE301) diluted to 4 to 40 units/ml with 10 mM Tris/HCl, pH 8.0, 0.1% Triton X-100, 0.5 mg/ml BSA. A dilution of 20 units/ml, assayed as described below, will produce an $A_{500\text{nm}}$ of ~ 0.5 .

Choline Solutions for Preparing Standard Curve (store at -20°C)

Concentrations of choline as desired from 0 to 6 mM in water.

Reaction Mix II (prepare fresh for assay and warm to 37°C just before use)

40 mM Tris/HCl pH 8.0; 2.4 mM EDTA; 0.6 mM 4-aminoantipyrine (added from 15 mM stock, stored at -20°C); 0.008% w/v phenol (added from 0.2% stock stored at -20°C); 8 units/ml choline oxidase (Sigma Cat. No. C5896); 8 units/ml peroxidase (Sigma Cat. No. P8250).

Choline Chloride Standard Solutions (in water)

For example, choline chloride solutions of 0.3, 0.6, 1.2, 1.8, 3.0, 4.8 and 6.0 mM, will, respectively, produce final concentrations of 5, 10, 20, 30, 50, 80, and 100 μM in the assay described below. For a 300 μl final assay volume in a standard 96-well plate, the optical path-length will be 0.8 cm. Choline added to a final concentration of 0.1 mM (100 μM) will produce 0.1 mM quinoneimine dye and an absorbance at 500 nm of: $12.2 \text{ mM}^{-1}\text{cm}^{-1} \times 0.1 \text{ mM} \times 0.8 \text{ cm} = 0.976$.

III. PROCEDURE

- Using a standard 96-well microtiter plate (flat-bottom, polystyrene), add 45 μl of 'Reaction Mix I' to all wells to be used in the assay (blank, standard or enzyme wells). Warm plate to 37°C .
- Add 5 μl of water to the wells that will serve as blanks. If preparing a choline standard curve, add 5 μl of the choline chloride standard solutions to the wells devoted to that.
- Initiate phospholipase D reactions by adding 5 μl of diluted enzyme to the appropriate wells and mixing. Start timing 10 min.
- Stop the phospholipase D reactions at 10 min. by addition of 250 μl 'Reaction Mix II'. Also add 250 μl 'Reaction Mix II' to blank and choline standard wells.

- E. Continue incubation at 37°C to allow the choline oxidase and peroxidase reactions to produce the colored reaction product. Color development should reach a maximum in 30-60 min., and will remain stable for at least 2-3 hr. afterwards.
- F. Quantitate the choline produced by the phospholipase D by comparison to the standard curve or use of the extinction coefficient for the quinoneimine dye ($12.2 \text{ mM}^{-1}\text{cm}^{-1}$).
- $\mu\text{mol choline/well} = (A_{500 \text{ nm}} \times 0.3 \text{ ml}) / (0.8 \text{ cm} \times 12.2 \text{ mM}^{-1}\text{cm}^{-1})$ **or** $[\text{choline}] (\text{mM}) \times 0.3 \text{ ml}$
- For a 10 min. reaction time, Phospholipase D Units/well = 6 x $\mu\text{mol choline/well}$

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