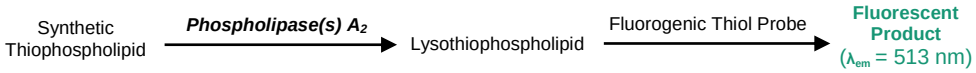
Phospholipase A_2 Activity Assay Kit (Fluorometric)

(Catalog # K400-100; 100 assays; Store at -20°C)

I. Introduction:

Phospholipase(s) A_2 (PLA_2s, EC 3.1.1.4) are a family of enzymes that catalyze the hydrolysis an acyl ester bond in the *sn*-2 position of a glycerophospholipid to release a lysophospholipid and a free fatty acid. Members of the PLA_2 superfamily can be divided into three major subtypes based upon their size and cation dependence. Two of the subtypes, the low molecular weight secretory phospholipase A_2 enzymes ($sPLA_2$) and the high molecular weight cytosolic enzymes ($cPLA_2$), are characterized by their requirement of Ca^{2+} for catalytic activity. The 14 kDa $sPLA_2$ enzymes are known to play an important role in the digestion of dietary phospholipids, host defense against bacterial infections as well as production of eicosanoids. The 85 kDa $cPLA_2$ enzymes play an important role in inflammatory disorders by releasing arachidonic acid from membrane phospholipids for production of various eicosanoids. The third subtype, the high molecular weight calcium-independent phospholipases A_2 ($iPLA_2$), are intracellular forms of the enzyme that have been recently implicated in functions such as phospholipid remodeling, cell proliferation and fat catabolism. BioVision's Phospholipase A_2 Activity Assay Kit provides a quick, sensitive and simple way for measuring PLA_2 activity in various biological samples. In this assay, active PLA_2 cleaves a synthetic thiophospholipid, producing a lysothiophospholipid which reacts with a fluorogenic probe to produce a highly fluorescent product detectable in the visible range (Ex/Em= 388/513 nm). The assay is simple to perform, high-throughput adaptable and can detect less than 0.1 mU of PLA_2 activity.



II. Applications:

- Measurement of phospholipase A_2 activity in various tissues/cells, venoms, secretory fluids.

III. Sample Types:

- Venoms and secretory fluids (snake venom, bee venom, synovial fluid, etc.)
- Soft tissue homogenates (liver, intestine, etc.)
- Cultured cells (adherent or suspension cells)

IV. Kit Contents:

Components	K400-100	Cap Code	Part Number
PLA_2 Assay Buffer	25 ml	WM	K400-100-1
PLA_2 Substrate	40 µl	Black	K400-100-2
PLA_2 Probe	100 µl	Red	K400-100-3
Fluorescence Standard	1 vial	Yellow	K400-100-4
Bee Venom Positive Control	1 vial	Violet	K400-100-5

V. User Supplied Reagents and Equipment:

- Black 96-well plate with flat bottom
- Multi-well spectrophotometer (ELISA reader)
- 30 kDa Spin Columns

VI. Storage Conditions and Reagent Preparation:

Store kit at -20°C, protected from light. Briefly centrifuge small vials prior to opening. Read entire protocol before performing the assay.

- **PLA_2 Assay Buffer:** Warm to room temperature before use. Store at 4°C or -20°C.
- **PLA_2 Substrate:** Allow to thaw to room temperature before use. Aliquot as desired and store at -20°C. While using, keep on ice after dilution. **Avoid repeated freeze/thaw cycles**. Use within two months.
- **PLA_2 Probe:** Allow to thaw to room temperature before use. Aliquot and store at -20°C.
- **Fluorescence Standard:** Reconstitute with 110 µl DMSO to generate 1 mM Standard solution. Aliquot and store at -20°C. Allow to thaw to room temperature before use and keep at room temperature while in use. Use within two months.
- **Bee Venom Positive Control:** Reconstitute with 22 µl PLA_2 Assay Buffer and mix thoroughly. Aliquot and store at **-80°C**. Use within two months. Keep on ice while in use.

VII. Secretory phospholipase A_2 Assay Protocol:

1. Sample Preparation:

a. Rapidly homogenize tissue (10 mg) or cells (1 x 10^6) with 100 µl ice-cold PLA_2 Assay Buffer and keep on ice for 10 min. Centrifuge at 10,000 x *g* for 10-15 min at 4°C and transfer the supernatant to a fresh, prechilled microfuge tube. Add 5-25 µl sample per well in a black 96 well plate and adjust the volume to 50 µl with PLA_2 Assay Buffer. For positive control: dilute the required amount of Bee Venom Positive Control 100 times with PLA_2 Assay Buffer. Add 10 µl of the diluted Bee Venom Positive Control per well into the desired well(s) and adjust the final volume to 50 µl with PLA_2 Assay Buffer. *Use the diluted positive control solution within one hour of preparation.*

Notes:

- Complex biological samples such as tissue homogenates and cell lysates may contain a mixture of PLA_2 subtypes; detected sample activity, therefore reflects total PLA_2 activity. The activity of low molecular weight (secretory) Phospholipase A_2 enzymes ($sPLA_2$s) may be determined by ultrafiltration of the sample using ***30 kDa MWCO*** spin columns to remove any high molecular

weight (cytosolic) phospholipase A_2 enzymes (cPLA$_2$s and iPLA$_2$s). Add sample to the spin column, centrifuge at 10,000 x *g* at 4°C for 10 min and collect the filtrate, which will contain only sPLA$_2$s.

- For unknown samples, we suggest doing a pilot experiment to ensure readings are within the range of the standard curve. Samples with extremely high PLA$_2$ activity (such as venoms and other secretory fluids) may be diluted with PLA$_2$ Assay Buffer.
- We recommend using a protease inhibitor cocktail (BioVision, Cat. # K271) to prevent enzyme degradation and measuring sample protein concentration using the Bradford reagent (BioVision, Cat. # K810 or a comparable protein assay).
- For samples exhibiting significant background, prepare parallel sample well(s) as background controls.

2. **Fluorescence Standard Curve:** Dilute the Fluorescence Standard stock solution 10 times with dH$_2$O just prior to use to obtain a 0.1 mM working solution. Add 0, 2, 4, 6, 8 and 10 µl of the 0.1 mM Fluorescence Standard into a series of wells in a 96 well plate to generate 0, 0.2, 0.4, 0.6, 0.8 and 1 nmol/well of Fluorescence Standard. Adjust the volume to 100 µl/well with PLA$_2$ Assay Buffer.
3. **Fluorogenic Probe Preparation:** Dilute the PLA$_2$ Probe stock solution 10 times with dH$_2$O to obtain a 1X working solution. Add 10 µl of the 1X working solution to all the wells except those containing the standards.
4. **Substrate Preparation and Reaction:** Dilute the PLA$_2$ Substrate stock solution 100 times with PLA$_2$ Assay Buffer to obtain a working solution. Make enough to add 40 µl per well (for example, for 10 reactions, add 4 µl of PLA$_2$ Substrate stock to 396 µl PLA$_2$ Assay Buffer to make 400 µl of working solution). Vortex and add 40 µl to each test and positive control well. *Do not add PLA$_2$ Substrate solution to the background control wells.*

	Test Sample/Positive Control	Sample Background Control
Substrate Solution (1X)	40 µl	—
PLA$_2$ Assay Buffer	—	40 µl

Note: The PLA$_2$ probe solution (1X) and the Substrate solution (1X) should be added to the wells sequentially as per protocol and not premixed as a single solution.

5. **Measurement:** Immediately begin measuring the fluorescence at Ex/Em= 388/513 nm in kinetic mode for 45-60 mins at 37°C. Measurement time for the linear phase of the reaction depends on the PLA$_2$ activity in samples. We recommend measuring the sample fluorescence in kinetic mode to ensure that the measurements recorded are within the linear range of the reaction.

   Note: The Fluorescence Standard Curve may be read separately in endpoint mode.

6. **Calculation:** Subtract the 0 nmol Fluorescence Standard reading from all standard curve readings, plot the standard curve and calculate the slope. For each reaction well (including sample background and positive control wells), choose two time points (t_1 and t_2) in the linear phase, obtain the corresponding fluorescence values at those points (RFU_1 and RFU_2) and determine the change in fluorescence over the time interval: $\Delta F = RFU_2 - RFU_1$. If sample background control reading is significant, subtract the background control reading from its paired sample reading. PLA$_2$ metabolic activity is obtained by applying the background-corrected ΔF values to the Fluorescence Standard curve to get *B* nmol of PLA$_2$ Substrate metabolized during the reaction time ($\Delta T = T_2 - T_1$).

Sample Phospholipase A_2 Activity = B/(ΔT X V) x D = nmol/min/ml = mU/ml

Where: **B** = metabolite amount from standard curve (nmol).
Δ**t** = reaction time of linear phase (min).
V = sample volume added into the reaction well (ml).
D = Sample dilution factor (if applicable)

Unit Definition: One unit of **Phospholipase A_2** is the amount of enzyme that generates 1.0 µmole of lysothiophospholipid metabolite per min at pH 7.5 at 37°C.

Note: Activity of the high molecular weight Phospholipase A_2 enzymes (cPLA$_2$ and iPLA$_2$ type enzymes) can be calculated by subtracting the activity of the secretory Phospholipase A_2 enzymes (filtered fraction) from the total Phospholipase A_2 activity.

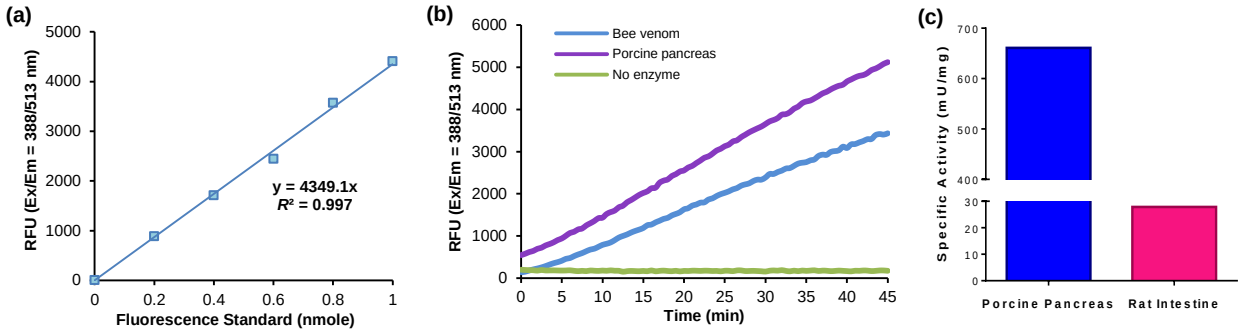


Figure: (a) Fluorescence standard curve. One mole of Fluorescence Standard corresponds to the metabolism of one mole of PLA$_2$ Substrate **(b)** Reaction kinetics of Phospholipase A_2 activity in bee venom positive control and porcine pancreas (0.314 µg protein) at 37°C using appropriate background controls. **(c)** Phospholipase A_2 specific activity calculated in porcine pancreas and rat intestine. Assays were performed following the kit protocol.

VIII. RELATED PRODUCTS:

Lipoprotein Lipase Activity Fluorometric Assay Kit (K721)
PAF Acetylhydrolase (PAF-AH) Inhibitor Screening Kit (K766)
Phospholipase D (PLD) Activity Assay Kit (K725)
Lipase Activity Colorimetric Assay Kit (K722)
Lipase Activity Fluorometric Assay Kit (K724)
PAF Acetylhydrolase (PAF-AH) Activity Assay Kit (K765)

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