ab133090 – Cytosolic Phospholipase A2 Assay Kit

Instructions for Use

For the detection of activity of $cPLA_2$ in purified preparations, cell cultures, or tissue homogenates.

This product is for research use only and is not intended for diagnostic use.

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1. Overview

ab133090 can be used to determine the activity of $cPLA_2$ in purified preparations, cell cultures, or tissue homogenates. Use of this assay with preparations containing more than one type of PLA_2 will result in the measurement of total PLA_2 activity rather than $cPLA_2$ alone.

2. Background

Phospholipases A2 (PLA_2s) catalyze the hydrolysis of fatty acids at the *sn*-2 position of glycerophospholipids, yielding a free fatty acid and a lysophospholipid as products. The release of arachidonic acid from membrane phospholipids by these enzymes is believed to be the key step in the biosynthesis of eicosanoids. There are primarily three different kinds of PLA_2s, they are secretory ($sPLA_2$), calcium-dependent cytosolic ($cPLA_2$), and calcium-independent ($iPLA_2$) PLA_2s. Of these three different types of enzymes, only the $cPLA_2$ exhibits specificity towards arachidonic acid whereas all others can hydrolyze any fatty acid at the *sn*-2 position.

Arachidonoyl thio-PC is a synthetic substrate that can be used to detect phospholipase activity. Hydrolysis of the arachidonoyl thioester bond at the *sn*-2 position by PLA_2 releases a free thiol which is detected by DTNB (5,5'-dithio-bis(2-nitrobenzoic acid); Ellman's reagent).

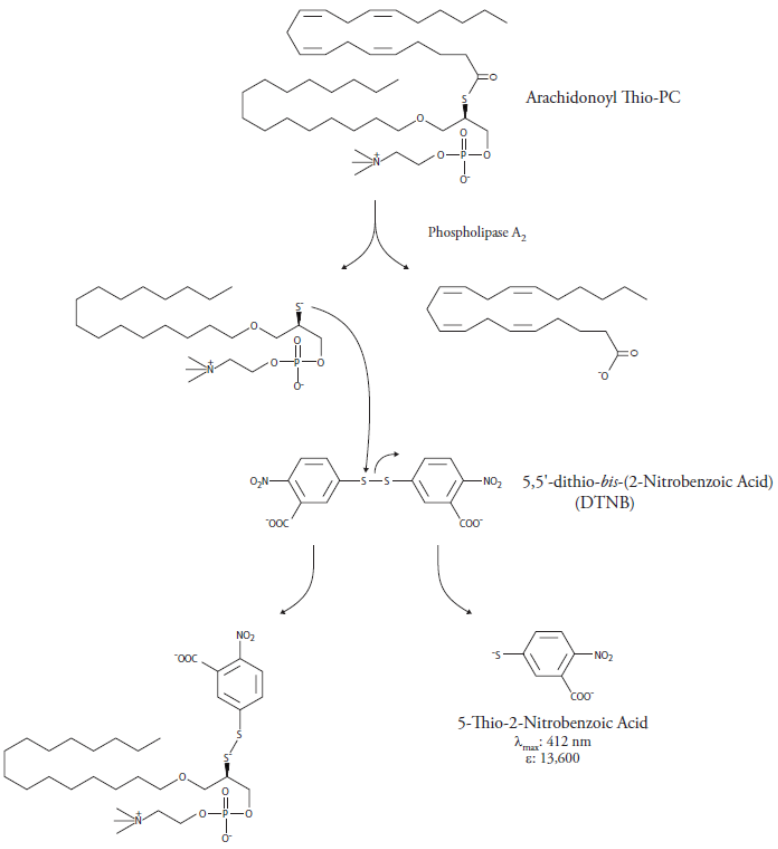

Figure 1. Assay scheme

3. Components and Storage

This kit will perform as specified if stored at -20°C.

Item	Quantity
$cPLA_2$ Assay Buffer	1 vial
DTNB/EGTA	4 vials
Arachidonoyl Thio-PC (Substrate)	2 vials
Bee venom PLA_2 Control	1 vial
Bromoenol Lactone Solution	1 vial
96-Well Solid Plate (Colorimetric Assay)	1 plate
96-well cover sheet	1 cover

Materials Needed But Not Supplied

- A plate reader capable of measuring absorbance at 414 or 405 nm.
- Adjustable pipettes and a repeat pipettor.
- A source of pure water; glass distilled water or HPLC-grade water is acceptable.

4. Pre-Assay Preparation

A. Reagent Preparation

Some of the kit components are in lyophilized form and need to be reconstituted prior to use. Follow the directions carefully to ensure proper volumes of water or Assay Buffer are used to reconstitute the components.

$cPLA_2$ Assay Buffer

The $cPLA_2$ Assay Buffer consists of 160 mM Hepes, pH 7.4, 300 mM NaCl, 20 mM $CaCl_2$, 8 mM Triton X-100, 60% glycerol, and 2 mg/ml BSA and should be used directly (without dilution) for the reconstitution of substrate. When stored at 4°C, this Assay Buffer is stable for at least one month. Dilute 5 ml of the Assay Buffer with 5 ml of HPLC-grade water and use this diluted Buffer in the assay, as well as, for dilution of samples prior to assaying.

DTNB/EGTA

Reconstitute the contents of one of the vials with 1.0 ml of HPLC-grade water to yield a mixture of 25 mM DTNB and 475 mM EGTA in 0.5 M Tris-HCl (pH 8.0). Store the reconstituted reagent on ice in the dark and use within eight hours.

Arachidonoyl Thio-PC (Substrate)

Evaporate the ethanolic solution of arachidonoyl Thio-PC to dryness under a gentle stream of nitrogen. NOTE: the substrate is supplied in ethanol containing 0.1% 2,6-Di-tert-butyl-4-methyl-phenol (BHT). Reconstitute the contents of each of the vials by vortexing with 6 ml of Assay Buffer (before dilution). Cool the vial on ice, vortex well until the substrate solution becomes clear, and then add 6 ml of HPLC-grade water to achieve a final concentration of 1.5 mM (high background absorbance may result if the substrate is not completely dissolved). The reconstituted substrate is stable for one week at -20°C.

Bee venom PLA_2 (Control)*

A 100 µg/ml solution of bee venom PLA_2 is supplied. To avoid repeated freezing and thawing, the PLA_2 can be aliquoted into several small vials. Bee venom PLA_2, when stored at -20°C, is stable for several months. Transfer 10 µl of the supplied enzyme to another vial and dilute with 490 µl of Assay Buffer (dilute) prior to use and use within one hour. A 10 µl aliquot of the enzyme per well causes a final absorbance of approximately 0.7 under the standard assay conditions described below.

*Since human cytosolic PLA_2 is not commercially available, bee venom PLA_2 is supplied as the positive control.

B. Sample Preparation

The sample must be free of particulates to avoid interferences in the absorbance measurement. Thiols, thiol-scavengers, and any endogenous PLA_2 inhibitors must be removed from the samples before performing the assay (extensive dialysis will eliminate most of the interfering substances of small molecular size).

If the samples are too dilute, they can be concentrated using a membrane filter with a molecular weight cut-off of 30,000 Da.

Tissue Homogenate

1. Prior to dissection, perfuse tissue with a PBS (phosphate buffered saline) solution, pH 7.4, containing 0.16 mg/ml heparin to remove any red blood cells and clots.

2. Homogenize the tissue in 5-10 ml of cold buffer (i.e., 50 mM Hepes, pH 7.4, containing 1 mM EDTA) per gram tissue. *NOTE: Tris or phosphate buffers can also be used.*

3. Centrifuge at 10,000 x g for 15 minutes at 4°C.

4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

Cell Lysate

1. Collect cells by centrifugation (i.e., 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.

2. The cell pellet can either be homogenized or sonicated in 1-2 ml of cold buffer (i.e., 50 mM Hepes, pH 7.4, containing 1 mM EDTA). *NOTE: Tris or phosphate buffers can also be used. Do not use SDS because it will interfere with the assay.*

3. Centrifuge at 10,000 x g for 15 minutes at 4°C.

4. Remove the supernatant for assay and store on ice. If not assaying on the same day, freeze the sample at -80°C. The sample will be stable for at least one month.

5. Assay Protocol

A. Plate Setup

There is no specific pattern for using the wells on the plate. However, it is necessary to have some wells (at least two) designated as non-enzyme controls or blank wells. The absorbance of these wells must be subtracted from the absorbance measured in the sample wells. We suggest that you have at least two wells designated as positive controls.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blk	Blk	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
B	+	+	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
C	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41
D	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34	S42	S42
E	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35	S43	S43
F	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36	S44	S44
G	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37	S45	S45
H	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38	S46	S46

Blk = Blank Wells

+ = Positive Control Wells

S1-S46 = Sample Wells

Pipetting Hints:

- *Use different tips to pipette substrate, DTNB/EGTA, and sample.*
- *Before pipetting each reagent, equilibrate the pipette tip in that reagent (i.e., slowly fill the tip and gently expel the contents, repeat several times).*
- *Do not expose the pipette tip to the reagent(s) already in the well.*

General Information:

- *The final volume of the assay is 225 µl in all wells.*
- *It is not necessary to use all the wells on the plate at one time.*
- *If the appropriate enzyme dilution is not known, it may be necessary to assay at several dilutions.*
- *Use the Assay Buffer (dilute) in the assay.*

B. Specificity

In general, any PLA_2 sample that can utilize arachidonoyl thio-PC as a substrate can be measured by this assay. Any residual $sPLA_2$ can be removed from the samples by using a membrane filter with a molecular weight cut-off of 30,000 or a $sPLA_2$ specific

inhibitor can be employed in the assay. To avoid any measurement of $iPLA_2$ activity in the sample, use the $iPLA_2$-specific inhibitor Bromoenol Lactone. Bromoenol Lactone should be added to the samples prior to assaying using the following procedure: Incubate sample (1 ml) with 5 µl of the 1 mM Bromoenol Lactone for 15 minutes at 25°C. The 5 µM final concentration of Bromoenol Lactone will inhibit any $iPLA_2$ present in the sample.

C. Performing the Assay

1. **Blank Wells (Non-enzymatic controls)** - Add 15 µl Assay Buffer to at least two wells (if performing inhibitor studies,* add 5 µl dimethylsulfoxide (DMSO) and 10 µl Assay Buffer instead of 15 µl Assay Buffer).

2. **Positive Control Wells (Bee venom PLA_2)** - add 10 µl PLA_2 and 5 µl Assay Buffer to at least two wells (if performing inhibitor studies,* add 5 µl DMSO instead of 5 µl Assay Buffer).

3. **Sample Wells** - add 10 µl sample and 5 µl Assay Buffer to at least three wells (if performing inhibitor studies,* add 5 µl of inhibitor dissolved in DMSO instead of 5 µl Assay Buffer). To obtain reproducible results, the amount of PLA_2 added to the well should result in a final absorbance between 0.4 and 1.2 or is at least 2-fold higher than the background absorbance. When

necessary, samples should be concentrated or diluted with Assay Buffer to bring the enzyme activity to this level. *NOTE: the amount of sample added to the well should always be 10 µl.*

4. Initiate the reactions by adding 200 µl substrate solution to all the wells. Carefully shake the plate for 30 seconds to mix and cover with the plate cover. Incubate for 60 minutes at room temperature.

5. Remove the plate cover. Add 10 µl of DTNB/EGTA to each well to stop enzyme catalysis and develop the reaction. Carefully shake the plate for 30 seconds to mix. Incubate for five minutes at room temperature.

6. Read the absorbance at 414 (or 405) nm using a plate reader.

*Inhibitors should be dissolved in DMSO and should be added to the assay in a final volume of 5 µl. In the event that the appropriate concentration of inhibitor is completely unknown, we recommend that several different dilutions of the inhibitor in DMSO be made.

6. Data Analysis

A. Calculations

Determination of PLA_2 Activity

1. Subtract the average absorbance of the non-enzymatic controls (Blanks) from the average absorbance of the samples and divide by the length of incubation (60 minutes).

$$A414/min = \frac{A414\ (sample) - A414\ (blank)}{60\ minutes}$$

2. Use the following formula to calculate the PLA_2 activity. The reaction rate at 414 nm can be determined using the DTNB extinction coefficient of 10.66 mM^{-1}. One unit of enzyme hydrolyzes one µmol of arachidonoyl Thio-PC per minute at 25°C.

$$cPLA_2\ Activity = \frac{\Delta A414/min}{10.66\ mM^{-1}} \times \frac{0.225\ ml \times Sample\ dilution}{0.01\ ml} = \mu mol/min/ml$$

*The actual extinction coefficient for DTNB at 414 nm is 13.6 $mM^{-1}cm^{-1}$. This value has been adjusted for the path length of the solution in the well (0.784 cm). The extinction coefficient for DTNB at 405 nm is 12.8 $mM^{-1}cm^{-1}$ and the adjusted value would be 10.0 mM^{-1}.

B. Performance Characteristics

Sensitivity: Samples containing $cPLA_2$ activity between 3.5-42 nmol/min/ml can be assayed without further dilution or concentration.

C. Interferences

Solvents: A slight decrease in enzymatic activity is observed when ethanol or methanol is added to the assay. The addition of DMSO has no effect on enzyme activity. PLA_2 inhibitors should be dissolved in DMSO and only 5 µl added to the assay.

Culture media and buffers: All buffers and media should be tested for high background absorbance before doing any experiments. If the initial background absorbances are higher than 0.3 absorbance units then the samples should be diluted in Assay Buffer (dilute) or water before performing the assay. Tris, Hepes, and phosphate buffers work in the assay.

Thiols and Thiol-scavengers: Samples containing thiols (i.e., glutathione, cysteine, dithiothreitol, or 2-mercaptoethanol) will exhibit high background absorbances and interfere with PLA2 activity determination. Samples containing thiol-scavengers (i.e., N-ethylmaleimide) will inhibit color development. Extensive dialysis will eliminate most of the interfering substances of small molecular size.

7. Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of Duplicates /triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No color development	A. DTNB or sample was not added to well(s) B. The enzyme activity was too low	A. Make sure to add all components to the wells and standardize the assay with bee venom PLA_2

Sample absorbance was over 1.2	The sample is too concentrated	Dilute the samples and re-assay
No activity was detected in the sample	It is possible that the sample was too dilute	Concentrate the sample using a membrane filter with a molecular weight cut-off of 30,000 and assay the concentrated sample again
High background absorbance	A. Substrate is not completely in solution B. Thiols present in sample	A. Make sure to vortex the substrate until a clear solution is made. B. Remove thiols or thiol reagents from sample

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