$sPLA_2$ (Type X) Inhibitor Screening Assay Kit

Item No. 702480

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -80°C kit. After opening kit, store individual components as stated below.

Item Number	Item	Quantity/Size	Storage
400587	$sPLA_2$ Assay Buffer (5X)	1 vial/12 ml	-20°C
400588	$sPLA_2$ (Type X) Substrate Resuspension Buffer	1 vial/8 ml	-20°C
400589	$sPLA_2$ (Type X) Substrate	1 vial/550 µl	-20°C
400590	$sPLA_2$ Assay Probe	1 vial/6 ml	-20°C
400591	$sPLA_2$ (Type X) Enzyme (human, recombinant)	1 vial/200 µl	-80°C
400592	$sPLA_2$ Control Inhibitor	1 vial/220 µl	-20°C
400593	384-Well Clear Bottom Black Plate	1 plate	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

This kit may not perform as described if any reagent or procedure is replaced or modified.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section (see page 3) and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure absorbance at a wavelength of 410 nm
2. Adjustable pipettes; multichannel or repeating pipettor recommended
3. An orbital microplate shaker
4. A source of pure water; glass-distilled water or deionized water is acceptable
   NOTE: UltraPure Water is available for purchase from Cayman (Item No. 400000)
5. Aluminum foil
6. DMSO

INTRODUCTION

Background

Secretory phospholipase A_2 ($sPLA_2$) (Type X) is a calcium-dependent PLA_2 superfamily member that is encoded by *PLA2G10* in humans.[1] It is expressed in the spleen, thymus, pancreas, lung, and colon, as well as peripheral blood leukocytes, neutrophils, and keratinocytes.[2-4] Upon activation, $sPLA_2$ (Type X) is released into the extracellular space where it acts in a paracrine or autocrine manner and preferentially catalyzes the hydrolysis of phosphatidylethanolamine (PE) and phosphatidylcholine (PC) over phosphatidylserine (PS) at the *sn-2* position, liberating the free fatty acid and lysophospholipid, which serve as substrates for the synthesis of bioactive lipid metabolites.[1,2] $sPLA_2$ (Type X) hydrolyzes lymphoma-derived extracellular vesicles (EVs), changing their size and morphology resulting in increased uptake by macrophages and increases the levels of EV-secreted lipid mediators.[5] Inhibition of $sPLA_2$ (Type X) reduces lymphoma growth in a mouse model of Epstein-Barr virus-induced B cell lymphoma, and $sPLA_2$ (Type X) levels in macrophages are negatively correlated with survival in patients with diffuse large B cell lymphoma (DLBCL). In addition, serum exosomal $sPLA_2$ (Type X) protein levels are increased in patients with non-small cell lung cancer (NSCLC).[6] $sPLA_2$ (Type X) levels are increased in sputum, as well as bronchial macrophages and columnar epithelial cells, in patients with asthma.[7] Based on these data, $sPLA_2$ (Type X) is a potential diagnostic and prognostic biomarker of NSCLC, and inhibition of $sPLA_2$ (Type X) is a potential therapeutic strategy in cancer and asthma.

About This Assay

Cayman's $sPLA_2$ (Type X) Inhibitor Screening Assay Kit provides a robust and easy-to-use platform for identifying novel inhibitors of human $sPLA_2$ (Type X), a phospholipase that hydrolyzes PE and PC at the *sn*-2 position. The assay uses a sulfur-containing analog of 1,2-diheptanoyl-*sn*-glycero-3-PC (di-C7-PC) as a substrate. Upon hydrolysis by $sPLA_2$ (Type X) at the *sn*-2 position, free thiols can be detected by the $sPLA_2$ Assay Probe resulting in a product that can be easily quantified using its absorbance at a wavelength of 410 nm. The potent and reversible $sPLA_2$ (Type X) inhibitor, $sPLA_2$ Control Inhibitor, also known as LY315920 or varespladib, is included as a positive control and inhibits the enzyme with an IC_{50} value of approximately 11 nM.

PRE-ASSAY PREPARATION

Sample Preparation

All inhibitors, be they small molecules, natural products, or proteins, should be prepared in a suitable solvent such as $sPLA_2$ Assay Buffer (1X), DMSO, dimethyl formamide (DMF), or short-chain alcohols (*e.g.*, MeOH, EtOH) at a concentration 35X the desired final assay concentration (*e.g.*, for 10 µM final assay concentration, a 350 µM stock should be made). The final concentration of the inhibitor diluent in the assay is 2.9% (see **Effects of Solvents** on page 17).

Reagent Preparation

1. **$sPLA_2$ Assay Buffer (5X) - (Item No. 400587)**

   Mix 10 ml of $sPLA_2$ Assay Buffer (5X) with 40 ml of pure water to make 50 ml of $sPLA_2$ Assay Buffer (1X). Once prepared, the $sPLA_2$ Assay Buffer (1X) may be stored at 4°C for at least three months.

2. **$sPLA_2$ (Type X) Substrate Resuspension Buffer - (Item No. 400588)**

   This vial contains 8 ml of $sPLA_2$ (Type X) Substrate Resuspension Buffer. Once thawed, it is ready to use as supplied. If all of the $sPLA_2$ (Type X) Substrate Resuspension Buffer will not be used at one time, aliquot and store it at -20°C.

3. **$sPLA_2$ (Type X) Substrate - (Item No. 400589)**

   This vial contains 13.75 mg of $sPLA_2$ (Type X) Substrate in 550 µl of ethanol (25 mg/ml concentration). For a full plate, completely evaporate all of the solvent under a gentle stream of inert gas (*e.g.* nitrogen, argon). The substrate will appear as a clear solid. Resuspend the substrate in 4.7 ml of $sPLA_2$ (Type X) Substrate Resuspension Buffer for a final concentration of 2.93 mg/ml.

   For a half a plate, aliquot 225 µl of substrate in solution, evaporate off the solvent, and resuspend in 2.35 ml of $sPLA_2$ (Type X) Resuspension Buffer. Unused substrate should be stored as an ethanol solution at -20°C, where it will be stable for at least six months. Resuspended substrate in $sPLA_2$ (Type X) Substrate Resuspension Buffer will be stable at room temperature for up to four hours, and at least two weeks at -20°C.

4. **$sPLA_2$ Assay Probe - (Item No. 400590)**

   This vial contains 6 ml of $sPLA_2$ Assay Probe. Mix 5 ml of $sPLA_2$ Assay Probe with 14 ml of $sPLA_2$ Assay Buffer (1X). Keep the diluted probe on ice until use. The diluted $sPLA_2$ Assay Probe will be stable for up to four hours on ice. It can be stored at -20°C for up to 3 months, limiting freeze-thaw cycles.

5. **$sPLA_2$ (Type X) Enzyme (human, recombinant) - (Item No. 400591)**

   $sPLA_2$ (Type X) Enzyme (human, recombinant) should be thawed on ice and gently mixed prior to dilution. To dilute the enzyme, mix 150 µl of $sPLA_2$ (Type X) Enzyme (human, recombinant) with 8.85 ml $sPLA_2$ Assay Buffer (1X). It is recommended that the enzyme be diluted immediately prior to performing the assay. The diluted enzyme loses 50% of its activity in one hour when stored on ice. The undiluted enzyme can be stored at -80°C, limiting freeze-thaw cycles.

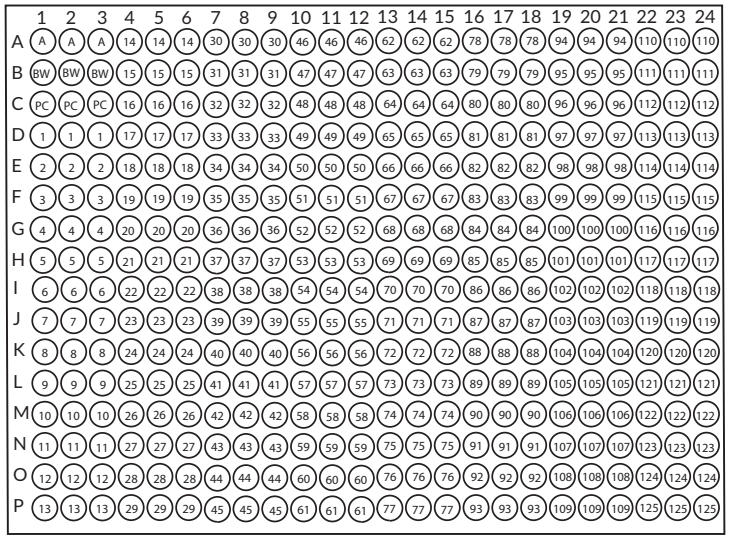
6. **$sPLA_2$ Control Inhibitor - (Item No. 400592)**

   This vial contains 200 µl of 1 mM $sPLA_2$ Control Inhibitor in DMSO, which can be used as a positive control. Mix 10 µl of $sPLA_2$ Control Inhibitor with 90 µl DMSO to make a 100 µM working solution. If all of the $sPLA_2$ Control Inhibitor will not be used at one time, aliquot the undiluted inhibitor and store at -20°C.

ASSAY PROTOCOL

Plate Set Up

The 384-well plate(s) included with this kit is supplied ready to use. There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% initial activity and three wells designated as background. It is suggested that each inhibitor, including the positive control $sPLA_2$ Control Inhibitor, be assayed at least in triplicate (quadruplicate preferred). A typical layout of samples to be measured in triplicate is shown in Figure 1, below.



A = 100% Initial Activity Wells
BW = Background Wells
PC - Positive Control Wells
1-125 = Sample Wells

Figure 1. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps maintain more precise incubation times.
- 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 70 µl in all the wells.
- All reagents should be prepared as described above. The $sPLA_2$ (Type X) enzyme should be kept on ice and all other reagents should be kept at room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended to assay the samples in triplicate (quadruplicate is preferred) but it is at the user's discretion to do so.
- The assay is performed at room temperature.
- Measure the absorbance at a wavelength of 410 nm.

Performing the Assay

1. **Background Wells**: add 38 µl of the diluted $sPLA_2$ Assay Probe, 20 µl of $sPLA_2$ Assay Buffer (1X), and 2 µl of solvent to three wells. Use the same solvent used for the unknown inhibitor and the positive control, $sPLA_2$ Control Inhibitor. If different solvents are to be assayed at the same time, separate sets of background wells should be run for each solvent.
2. **100% Initial Activity Wells**: add 38 µl of the diluted $sPLA_2$ Assay Probe, 20 µl of the diluted $sPLA_2$ (Type X) Enzyme, and 2 µl of solvent to three wells. Use the same solvent used for the unknown inhibitor and the positive control, $sPLA_2$ Control Inhibitor. If inhibitors in different solvents are to be assayed at the same time, separate sets of 100% initial activity wells should be set up for each solvent.
3. **Inhibitor/Positive Control Wells**: add 38 µl of the diluted $sPLA_2$ Assay Probe, 20 µl of the diluted $sPLA_2$ (Type X) Enzyme, and 2 µl of unknown inhibitor or the 100 µM positive control, $sPLA_2$ Control Inhibitor working solution, to three wells.
4. Incubate for 15 minutes at room temperature.
5. Initiate the reactions by adding 10 µl of reconstituted $sPLA_2$ (Type X) Substrate to all the wells being used. Mix on an orbital microplate shaker for one minute.
6. Cover the plate with foil and incubate for 60 minutes at room temperature.
7. Remove the foil and read the absorbance at a wavelength of 410 nm.

NOTE: If desired, the assay may be read kinetically rather than as an endpoint. If reading kinetically, skip step #6 above. The absorbance should be measured at least once per minute at room temperature for 60 minutes. Determine the initial rate based on the linear portion of the kinetic curve. Calculations can be performed as shown below substituting initial rates for average absorbance.

ANALYSIS

Calculations

1. Determine the average absorbance of each sample.
2. Subtract the average absorbance of the background wells from the average absorbance of the 100% initial activity and inhibitor wells. These are the corrected values.
3. Determine the percent inhibition or percent activity for each inhibitor using one of the following equations:

$$\% \text{ inhibition} = \left[\frac{\text{corrected 100\% initial activity - corrected inhibitor activity}}{\text{corrected 100\% initial activity}} \right] \times 100$$

$$\% \text{ activity} = \left[\frac{\text{corrected inhibitor activity}}{\text{corrected 100\% initial activity}} \right] \times 100$$

4. Graph the percent inhibition or percent activity as a function of inhibitor concentration to determine the IC_{50} value (the concentration at which there is 50% inhibition) of the inhibitor. Inhibition of recombinant human $sPLA_2$ (Type X) by $sPLA_2$ Control Inhibitor is shown in Figure 2 (see page 15).

Performance Characteristics

Z´ Factor:

Z´ factor is a term used to describe the robustness of an assay, which is calculated using the equation below.[8]

$$Z' = 1 - \frac{3\sigma_{c+} + 3\sigma_{c-}}{| \mu_{c+} - \mu_{c-} |}$$

Where σ: Standard deviation
μ: Mean
c+: Positive control
c-: Negative control

The theoretical upper limit for the Z' factor is 1.0. A robust assay has a Z' factor >0.5. The Z' factor for Cayman's $sPLA_2$ (Type X) Inhibitor Screening Assay Kit was determined to be 0.91.

Sample Data:

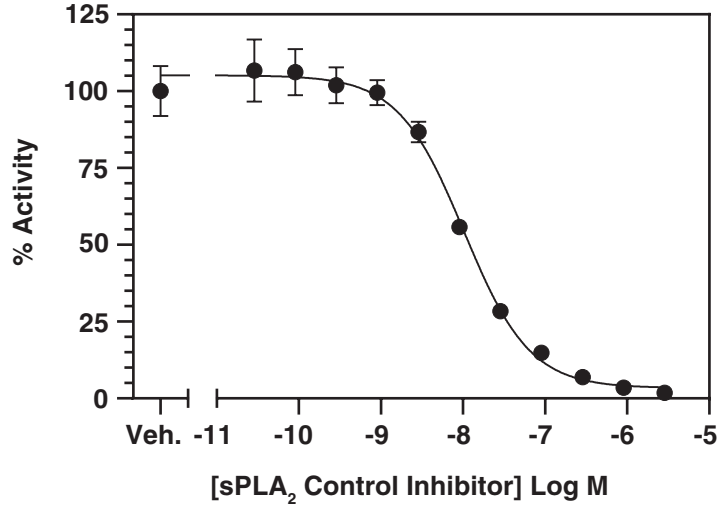


Figure 2. Inhibition of recombinant human $sPLA_2$ (Type X) by $sPLA_2$ Control Inhibitor. Data are plotted as the mean of triplicate measurements ± the standard deviation. The vehicle control (Veh.) represents 100% initial activity. The IC_{50} value of $sPLA_2$ Control Inhibitor is 11 nM.

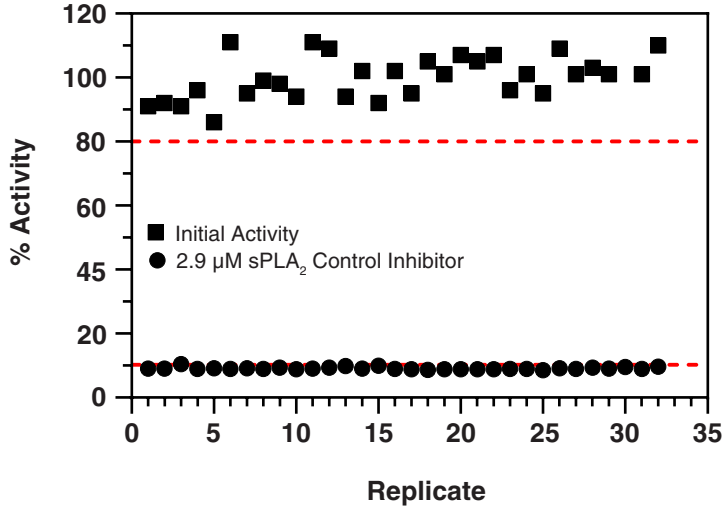


Figure 3. Typical performance data for the sPLA$_2$ (Type X) Inhibitor Screening Assay Kit. Data are shown from 32 replicates each for 100% initial activity wells and 2.9 µM sPLA$_2$ Control Inhibitor prepared as described in the kit booklet. The calculated Z′ factor for this experiment was 0.91. The red lines correspond to three standard deviations from the mean for each control value.

Effects of Solvents:

Compounds may be prepared in organic solvents such as DMSO, DMF, or short-chain alcohols (*e.g.*, MeOH, EtOH). The final concentration of the inhibitor diluent in the assay is 2.9%. A titration of organic solvents showed that signal can change with increasing solvent concentration, so the proper vehicle control should be included in the assay.

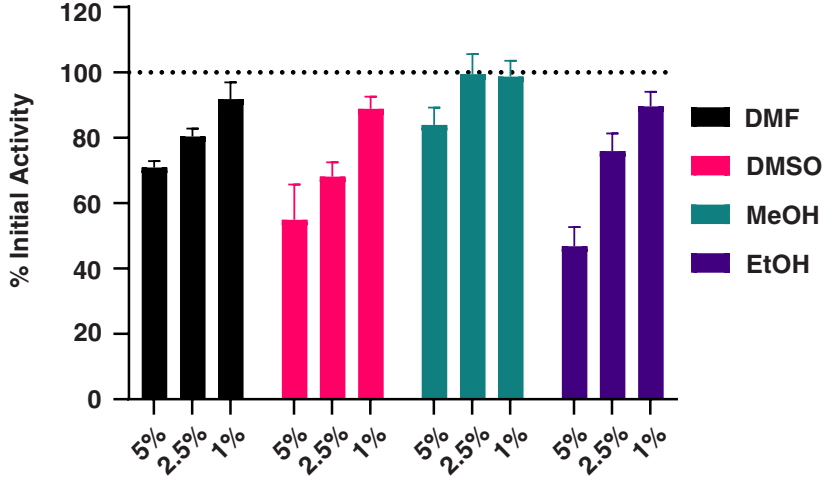


Figure 4. The effect of solvent on the readout of sPLA$_2$ (Type X) activity. The data are shown as the mean ± standard deviation for quadruplicate reactions containing the indicated concentration of solvents. The dotted line represents sPLA$_2$ (Type X) activity in sPLA$_2$ Assay Buffer (1X).

Precision:

Intra-assay precision was determined by analyzing 15 measurements of the background, 100% initial activity, and 2.9 µM $sPLA_2$ Control Inhibitor on the same day. The intra-assay coefficients of variation were 7, 7, and 2%, respectively. The intra-assay coefficient of variation for the IC_{50} value of 15 inhibition curves performed on the same day was 15%.

Inter-assay precision was determined by analyzing inhibition with $sPLA_2$ Control Inhibitor in six separate assays on three different days. The inter-assay coefficient of variance for the IC_{50} value was 14%.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of triplicates/ quadruplicates	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 absorbance above background is seen in the Inhibitor wells	A. Enzyme, probe, or substrate was not added to the well(s) or the enzyme has degraded. B. Inhibitor concentration is too high and inhibited all of the enzyme activity	A. Make sure to add all components to the wells. Use the diluted enzyme immediately or store it on ice for not more than 1 hour. B. Reduce the concentration of the inhibitor and re-assay
No inhibition seen with inhibitor	A. The inhibitor concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay

Reagent	Procedure
$sPLA_2$ Assay Buffer (5X)	Dilute 1:5 with pure water to make Assay Buffer (1X)
$sPLA_2$ (Type X) Substrate Resuspension Buffer	Ready to use as supplied
$sPLA_2$ (Type X) Substrate	1. Evaporate ethanol under a gentle stream of inert gas 2. Resuspend the substrate in 4.7 ml of $sPLA_2$ (Type X) Substrate Resuspension Buffer or resuspend the amount needed to a final concentration of 2.93 mg/ml
$sPLA_2$ Assay Probe	Dilute 5 ml of $sPLA_2$ Assay Probe with 14 ml of $sPLA_2$ Assay Buffer (1X)
$sPLA_2$ (Type X) Enzyme	Dilute 1:60 with $sPLA_2$ Assay Buffer (1X)
$sPLA_2$ Control Inhibitor	Dilute to 1:10 with DMSO to make a 100 µM inhibitor working solution

Table 1. Assay reagent preparation summary

Procedure	Background Wells	100% Activity Wells	Positive Control Wells	Inhibitor Wells
Add diluted $sPLA_2$ Assay Probe	38 µl	38 µl	38 µl	38 µl
Add $sPLA_2$ Assay Buffer (1X)	20 µl	--	--	--
Add diluted $sPLA_2$ (Type X) Enzyme	--	20 µl	20 µl	20 µl
Add solvent	2 µl	2 µl	--	--
Add 100 µM $sPLA_2$ Control Inhibitor	--	--	2 µl	--
Add Test Inhibitor (35X)	--	--	--	2 µl
Incubate	Incubate for 15 minutes at room temperature.			
Add reconstituted $sPLA_2$ (Type X) Substrate	10 µl	10 µl	10 µl	10 µl
Mix, Incubate, and Read	Mix on an orbital microplate shaker for 1 minute. Cover with foil and incubate at room temperature for 60 minutes. Read absorbance at 410 nm.			

Table 2. Assay summary

References

1. Murakami, M., Taketomi, Y., Sato, H., *et al.* Secreted phospholipase A_2 revisited. *J. Biochem.* **150(3)**, 233-255 (2011).
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8. Zhang, J.-H., Chung, T.D.Y., and Oldenburg, K.R. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* **4(2)**, 67-73 (1999).

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.