$sPLA_2$ (Type V) Inhibitor Screening Assay Kit (PC Substrate)

Item No. 10004883

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1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

Item Number	Item	Quantity
765010	$sPLA_2$ Assay Buffer (10X)	1 vial
765012	$sPLA_2$ DTNB	4 vials
765015	$sPLA_2$ Diheptanoyl Thio-PC (substrate)	2 vials
10004913	$sPLA_2$ Assay (human Type V)	1 vial
765017	$sPLA_2$ Positive Control Inhibitor	1 vial
400014	96-Well Solid Plate (Colorimetric Assay)	1 plate
400012	96-Well Cover Sheet	1 cover

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.

If You Have Problems

Technical Service Contact Information

Phone:	888-526-5351 (USA and Canada only) or 734-975-3888
Fax:	734-971-3641
E-Mail:	techserv@caymanchem.com
Hours:	M-F 8:00 AM to 5:30 PM EST

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 at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader capable of measuring absorbance between 405-420 nm
2. Adjustable pipettes and a repeating pipettor
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of phospholipids at the *sn*-2 position yielding a free fatty acid and a lysophospholipid.[1] The release of arachidonic acid from membrane phospholipids by PLA_2 is believed to be a key step in the control of eicosanoid production within the cell.[2] Secretory PLA_2s ($sPLA_2$s) are proteins of relatively low molecular mass (about 14 kDa), are highly enriched in disulfide bonds, and require millimolar levels of Ca^{2+} for activity. Mammalian tissues contain many $sPLA_2$s, including Types IB, IIA-F, III, V, X, and XII.[3] Type V $sPLA_2$ has been shown to be involved in eicosanoid formation in inflammatory cells, such as macrophages and mast cells.[4,5] It has been demonstrated that human Type V $sPLA_2$ can bind phosphatidylcholine (PC) membranes and hydrolyze PC substrates much more efficiently than human Type IIA $sPLA_2$, which makes it better suited for acting on the outer plasma membrane.[6]

About This Assay

Selective targeting and inhibition of $sPLA_2$s has been problematic as evidenced by the lack of availability of isozyme-specific inhibitors. Cayman's $sPLA_2$ (Type V) Inhibitor Screening Assay Kit (PC Substrate) provides a convenient method for screening Type V $sPLA_2$ inhibitors. The assay utilizes the 1,2-dithio analog of diheptanoyl phosphatidylcholine as a substrate.[7,8] Upon hydrolysis of the thio ester bond at the *sn*-2 position by $sPLA_2$, free thiols are detected using DTNB (5,5'-dithio-*bis*-(2-nitrobenzoic acid); Ellman's reagent; see Scheme 1, on page 7). The $sPLA_2$ (Type V) Inhibitor Screening Assay Kit (PC Substrate) includes human recombinant Type V $sPLA_2$ and is a time saving tool for screening numerous inhibitors. Identification of selective $sPLA_2$ inhibitors will offer substantial aid in the elucidation of the specific physiological function of each $sPLA_2$.

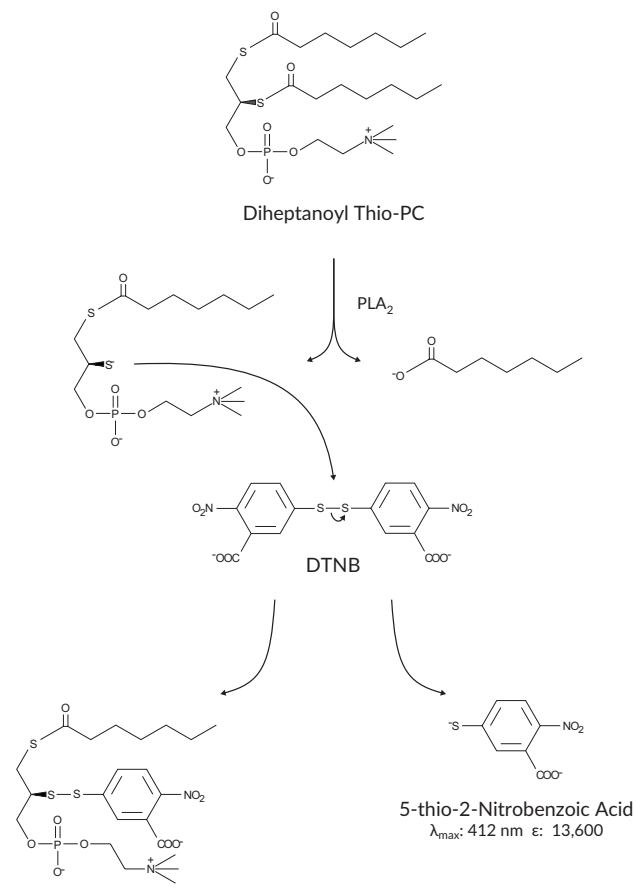


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

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

1. **Assay Buffer (10X) - (Item No. 765010)**

   Dilute 3 ml of Assay Buffer concentrate with 27 ml of HPLC-grade water. This final Assay Buffer (25 mM Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$, 100 mM KCl, and 0.3 mM Triton X-100) should be used for reconstitution of substrate and dilution of water-soluble inhibitors. When stored at 4°C, this diluted Assay Buffer is stable for at least two months.

2. **DTNB - (Item No. 765012)**

   Reconstitute the contents of one vial with 1.0 ml of HPLC-grade water to yield 10 mM DTNB in 0.4 M Tris-HCl, pH 8.0. Store the reconstituted reagent on ice in the dark and use within eight hours.

3. **Diheptanoyl Thio-PC (Substrate) - (Item No. 765015)**

   Evaporate the ethanolic solution of diheptanoyl thio-PC to dryness under a gentle stream of inert gas (*e.g.*, nitrogen, argon). Reconstitute the contents of each vial with 12 ml of diluted Assay Buffer to achieve a concentration of 1.66 mM. Make sure to vortex until the Substrate Solution becomes clear. The Substrate, when stored at -20°C in Assay Buffer, is stable for at least two weeks. *NOTE: If not using the entire plate, reconstitute only one of the Substrate vials. The K_M value for diheptanoyl thio-PC is 0.78 mM for human (Type V) $sPLA_2$. The final concentration of diheptanoyl-thio PC in the assay as described below is 1.44 mM. This concentration may be reduced with Assay Buffer at the users discretion, particularly when complete inhibition curves are required for IC_{50} or K_i determination. For competitive inhibitors the IC_{50} is dependent upon the Substrate concentration and should be reported when publishing the experimental results. An example is exhibited in Figure 3, on page 14, using the competitive $sPLA_2$ inhibitor, thioetheramide-PC.*

4. **Human (Type V) $sPLA_2$ - (Item No. 10004913)**

   The vial contains a solution of human recombinant (Type V) $sPLA_2$. The thawed enzyme should be stored on ice. Dilute 20 µl of enzyme with 980 µl of diluted Assay Buffer. The diluted enzyme is stable for four hours on ice.

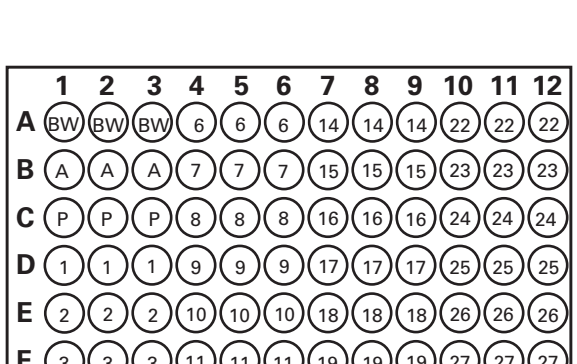
5. **$sPLA_2$ Positive Control Inhibitor - (Item No. 765017)**

   The vial contains a 2.3 mM solution of Thioetheramide-PC dissolved in ethanol.

ASSAY PROTOCOL

Plate Set Up

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, three wells designated as positive control inhibitor wells, and three wells designated as background wells. A typical layout of samples and inhibitors to be measured in triplicate is given below in Figure 2. We suggest that each inhibitor sample be assayed in triplicate and that you record the contents of each well on the template sheet provided on page 18.



BW - Background Wells
A - 100% Initial Activity Wells
P - Positive Control Inhibitor Wells
1-29 - Test Compound Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps to maintain more precise incubation times.
- Use different tips to pipette the enzyme, DTNB, and Substrate.
- 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 230 µl in all wells.
- It is not necessary to use all the wells on the plate at one time.
- The assay temperature is 25°C.
- If the appropriate inhibitor concentration is not known, it may be necessary to assay at several dilutions.
- We recommend assaying samples in triplicate, but it is the user's discretion to do so.
- Twenty-nine inhibitor samples can be assayed in triplicate or forty-five in duplicate.
- Monitor the absorbance at 405-420 nm using a plate reader.

Performing the Assay

1. **100% Initial Activity Wells** - add 10 µl of $sPLA_2$ and 10 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells. The 100% initial activity wells should exhibit an absorbance of ~0.8-0.9.
2. **Positive Control Inhibitor Well** - add 10 µl of sPLA2 and 10 µl of Thioetheramide-PC to three wells. The final concentration of Thioetheramide-PC in the positive control wells is 100 µM.
3. **Inhibitor Wells** - add 10 µl of $sPLA_2$ and 10 µl of inhibitor* to three wells.
4. **Background Wells** - add 10 µl of Assay Buffer and 10 µl of solvent (the same solvent used to dissolve the inhibitor) to three wells.
5. Add 200 µl of Substrate Solution to all the wells being used.
6. Add 10 µl of DTNB to all wells to initiate the reaction.
7. Carefully shake plate for 10 seconds to mix and then cover with plate cover. Incubate for 15 minutes at 25°C.
8. Read at an absorbance between 405-420 nm using a plate reader. Assay can also be read kinetically after initiation with DTNB, and the rate used to determine the IC_{50} of the inhibitor.

*Inhibitors can be dissolved in methanol, dimethyl sulfoxide, or ethanol and should be added to the assay in a final volume of 10 µl. In the event that the appropriate concentration of inhibitor needed for $sPLA_2$ inhibition is completely unknown, we recommend that several dilutions of the inhibitor be assayed.

ANALYSIS

Calculations

1. Determine the average absorbance of each sample.
2. Subtract the absorbance of the background wells from the absorbance of the 100% initial activity and the inhibitor wells.
3. Determine the percent inhibition for each sample. To do this, subtract each inhibitor sample value from the 100% initial activity sample value. Divide the result by the 100% initial activity value and then multiply by 100 to give the percent inhibition.
4. Either graph the Percent Inhibition or Percent Initial Activity as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition). An example of human recombinant Type V $sPLA_2$ inhibition by Thioetheramide-PC is shown in Figure 3.

Sample Data

The standard curve presented here is an example of the data typically produced with this kit; however, your results will not be identical to these. Do not use the data below to determine the values of your samples. Your results could differ substantially.

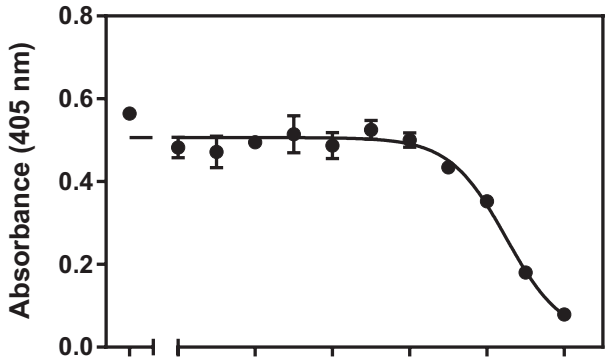


Figure 3. Inhibition of human (Type V) $sPLA_2$ by Thioetheramide-PC. The IC_{50} range for a typical Thioetheramide-PC inhibition curve should fall between 12.5 and 19.1 µM. "Veh." represents compound vehicle control.

RESOURCES

Interferences

Inhibitors containing thiols will exhibit high absorbance due to the direct reaction with DTNB. Inhibitors that are thiol-scavengers will inhibit color development and interfere with $sPLA_2$ activity determination.

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 absorbance above 0.1 is seen in the Inhibitor wells	A. Enzyme, DTNB, or substrate was not added to the well(s) B. Inhibitor concentration is too high and inhibited all of the enzyme activity	A. Make sure to add all components to the wells 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

References

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5. Han, S.-K., Yoon, E.T., and Cho, W. Bacterial expression and characterization of human secretory class V phospholipase A_2. *Biochem. J.* **331**, 353-357 (1998).
6. Han, S.K., Kim, K.P., Koduri, R., *et al.* Roles of Trp31 in high membrane binding and proinflammatory activity of human group V phospholipase A_2. *J. Biol. Chem.* **274**, 11881-11888 (1999).
7. Hendrickson, H.S., Kendrickson, E.K., and Dybvig, R.H. Chiral synthesis of a dithiolester analog of phosphatidylcholine as a substrate for the assay of phospholipase A_2. *J. Lipid Res.* **24**, 1532-1537 (1983).
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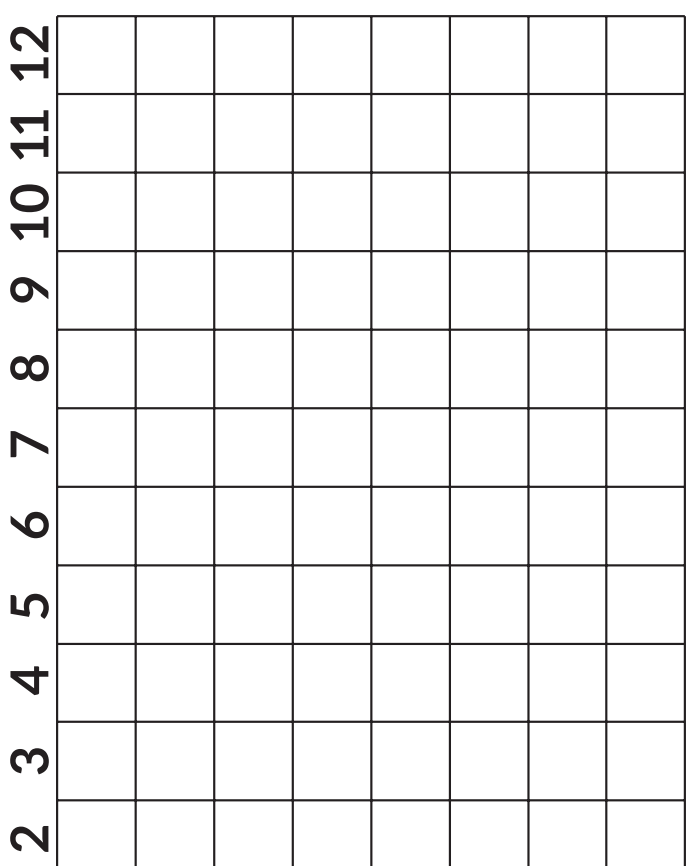

NOTES

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.