Fluorogenic Phospholipase A Substrates

Catalog nos. A10070, A10072, B7701, D23739

Table 1. Contents and storage information.

Material	Amount	Storage	Stability
PED-A1 (A10070) N-((6-(2,4-DNP)amino)hexanoyl)-1-(BODIPY®FL C_5)-2-hexyl-*sn*-glycero-3-phosphoethanolamine	100 µg	• ≤–20ºC • Desiccate • Protect from light	When stored as directed the product is stable for at least 1 year.
Red/Green BODIPY® PC-A2 (A10072) 1-*O*-(6-BODIPY® 558/568-aminohexyl)-2-BODIPY® FL C_5-*sn*-glycero-3-phosphocholine	100 µg		
bis-BODIPY® FL C_{11}-PC (B7701) 1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a, 4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero- 3-phosphocholine	100 µg		
PED6 (D23739) *N*-((6-(2,4-dinitrophenyl) amino)hexanoyl)-2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl- *sn*-glycero-3-phosphoethanolamine, triethylammonium salt	1 mg		
Approximate fluorescence excitation and emission maxima: See Table 2.			

Introduction

The importance of phospholipases in cellular signaling, lipid metabolism, inflammatory responses, and pathological disorders related to these processes has stimulated demand for fluorescence-based enzyme activity monitoring methods.[1-6] Fluorogenic phospholipase A substrates from Invitrogen are designed to provide continuous monitoring of phospholipase A (PLA) activity in purified enzyme preparations, cell lysates, and living cells. Indeed, applications of these substrates extend as far as *in vivo* small animal imaging.[7] The PLA substrates are dye-labeled phospholipids of two types—glycerophosphocholines with BODIPY® dye-labeled *sn*-1 and *sn*-2 acyl or alkyl chains (Figure 1) and glycerophosphoethanolamines with BODIPY® dye-labeled acyl chains and dinitrophenyl quencher-modified head groups (Figure 2). These structural variations determine specificity for PLA_1 versus PLA_2 and the fluorescence response associated with enzymatic cleavage of the substrate, as summarized in Table 1.

Red/Green BODIPY® PC-A2 provides a capacity for dual emission fluorescence ratio detection that is not afforded by other fluorogenic phospholipase substrates (Figure 3). Cleavage of the BODIPY® FL pentanoic acid substituent at the *sn*-2 position results in decreased quenching by fluorescence resonance energy transfer (FRET) to the BODIPY® 558/568 dye attached to the *sn*-1 position (Figure 1B). The result is a PLA_2-dependent increase in BODIPY® FL fluorescence emission detected in the range 515-545 nm. The FRET-sensitized BODIPY® 558/568 fluorescence signal is expected to show a reciprocal decrease. In practice, BODIPY® 558/568 fluorescence may show a decrease or a slight increase (Figure 3B), depending on the formulation of the substrate and the instrument excitation/emission wavelength settings. The dual emission properties of this substrate also provide the capacity to localize the lysophospholipid and fatty acid products of PLA_2 cleavage via their distinct spectroscopic signatures in imaging experiments.

Table 2. Fluorogenic phospholipase A substrates.

Substrate	Cat. no.	Specificity	Structure	Cleavage Product*	Detection**
bis-BODIPY® FL C_{11}-PC	B7701	PLA_1 or PLA_2	Figure 1 A	D3862	Fluorescence intensity increase, Ex = 488 nm, Em 530 nm
Red/Green BODIPY® PC-A2	A10072	PLA_2	Figure 1 B	D3834	Fluorescence emission ratio increase, Ex = 488 nm, Em = 530/590 nm#
PED6	D23739	PLA_2***	Figure 2 A	D3834	Fluorescence intensity increase, Ex = 488 nm, Em = 530 nm
PED-A1	A10070	PLA_1	Figure 2 B	D3834	Fluorescence intensity increase, Ex = 488 nm, Em = 530 nm

*Catalog number of the fatty acid product generated by action of the enzyme listed in column 3. These materials are useful as standards for evaluating percentage substrate conversion in enzymatic reactions. **Fluorescence signal corresponding to increasing phospholipase A activity. Ex = excitation wavelength, Em = emission wavelength. For detection, closely match (does not need to be exact) the instrument settings to these wavelength specifications. ***PED6 may be cleaved by PLA_1 but this reaction does not result in separation of the fluorophore and quencher substituents. #May also be monitored via fluorescence intensity increase Ex = 488 nm, Em 530 nm if ratiometric readout is not desired.

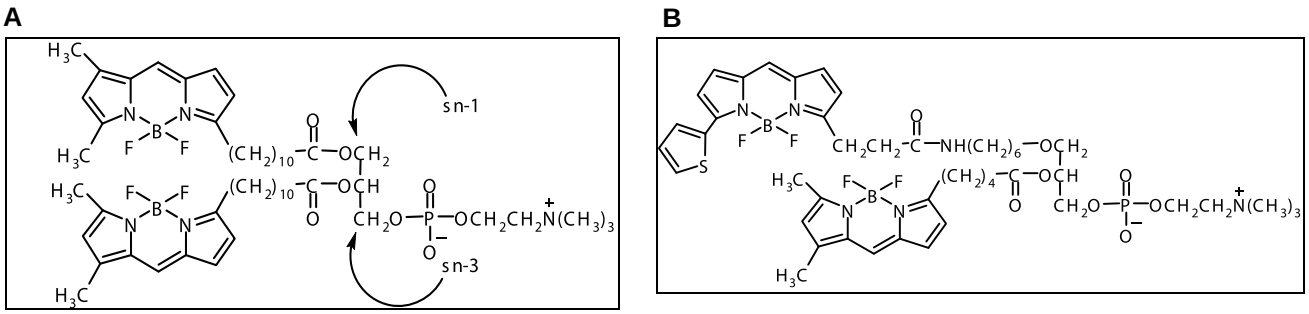

Figure 1. Panel A: Bis-BODIPY® FL C_{11}-PC .Panel B: Red/Green BODIPY® PC-A2.

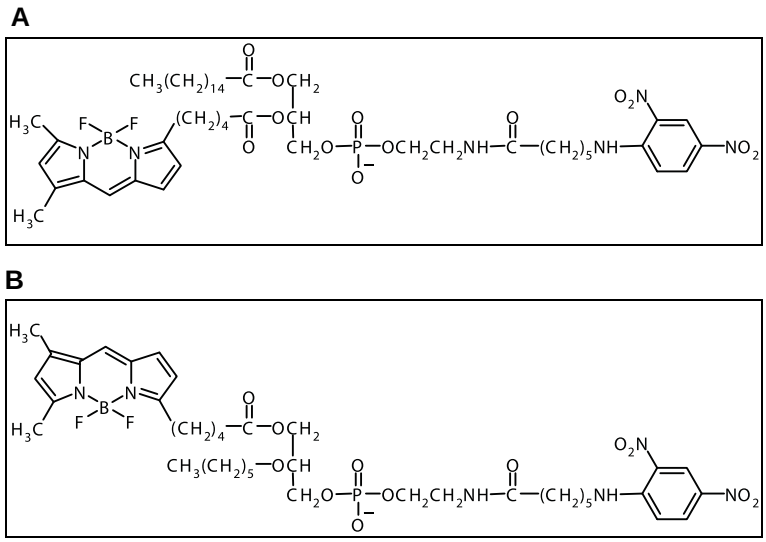

Figure 2. Panel A: PED6. Panel B: PED-A1.

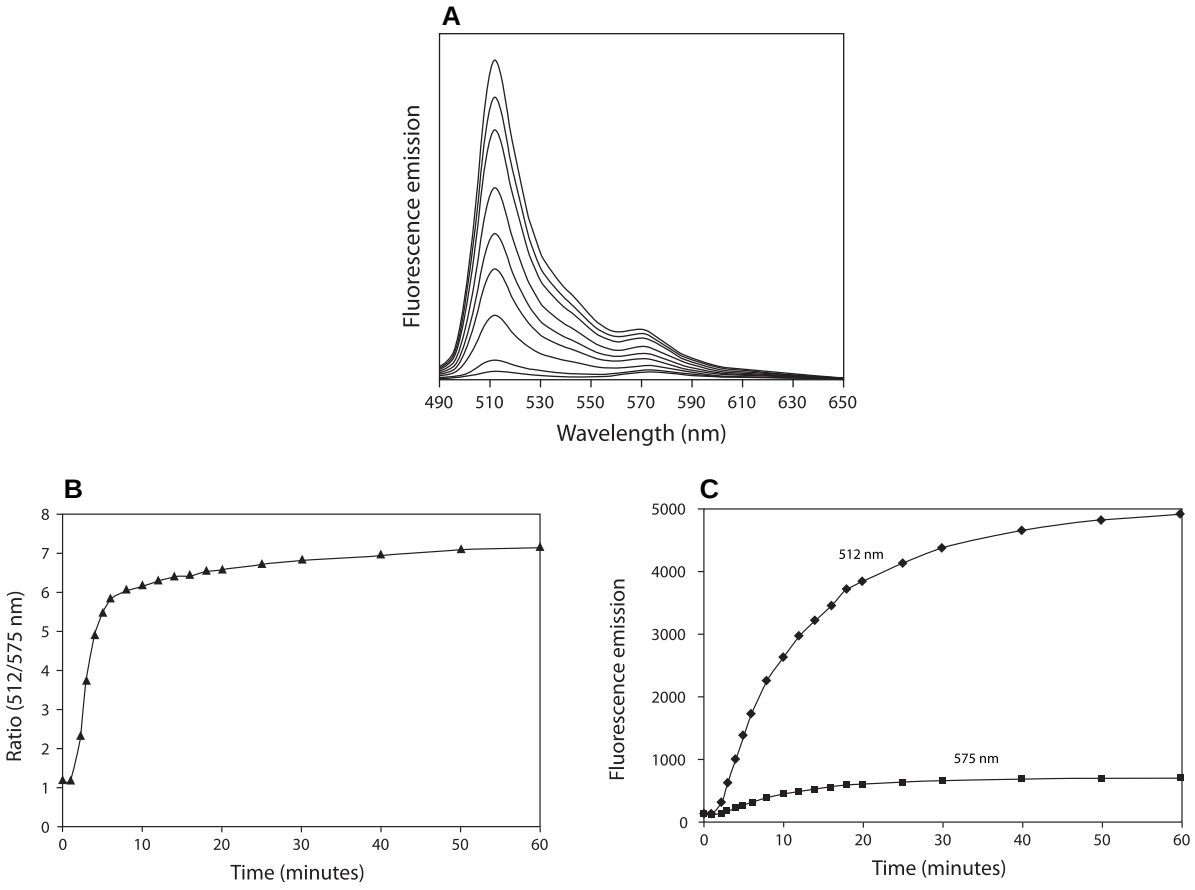

Figure 3. Panel A: Fluorescence emission spectra (excitation at 480 nm) of Red/Green BODIPY® PC-A2 (5 µM) incorporated in liposomes containing 25 µM DOPC and 25 µM DOPG. In descending order, the topmost seven nested curves represent time points 58, 28, 18, 10, 6, 4, and 2 minutes after addition of 0.5 units of bovine pancreatic PLA_2. The two curves closest to the x-axis baseline represent 15 seconds after enzyme addition and 2 minutes before addition. Panel B. Plot of the ratio of green (512 nm) to red (575 nm) fluorescence emission intensities versus time derived from data shown in panel A. PLA_2 addition at t = 2 minutes. Panel C: Plot of green (512 nm) and red (575 nm) fluorescence emission intensities versus time derived from data shown in panel A. PLA_2 addition at t = 2 minutes.

Before You Begin

Materials Required but Not Provided

Use the amounts of material specified as guidelines only, and scale up or down depending on experimental requirements.

For Analysis of nonadherent cells by flow cytometry

- Phosphatidylserine (PS; Avanti Polar Lipids, Alabaster, AL or Sigma Chemical Co., St. Louis, MO)
- Sterile, tissue culture grade, phosphate buffered saline (PBS; Invitrogen Cat. no. 20012-027)
- Dry chloroform
- Probe-type sonicator capable of delivering power outputs up to 100 W into a 1–2 mL sample

For PLA_2 Fluorescence microplate assay

- Phospholipase A_2

  Note: This protocol was developed and tested using group IB PLA_2 from bovine pancreas and group III PLA_2 from bee venom.
- Dioleoylphosphatidylcholine (DOPC; Avanti Polar Lipids, Alabaster, AL)
- Dioleoylphosphatidylglycerol (DOPG; Avanti Polar Lipids, Alabaster, AL)
- Ethanol, DMSO (dimethylsulfoxide)
- Magnetic stirrer, stir bar, and pipettor (an air displacement pipettor with 100 µL capacity, fitted with a narrow orifice gel-loading tip is suitable and is required for liposomes preparation by ethanol injection at step 3.4)

Preparing Stock Solutions

1.1 Prepare 1 mM (approximately 1 mg/mL) stock solutions of bis-BODIPY® FL C_{11}-PC and Red/Green BODIPY® PC-A2 by dissolving:

- 100 µg bis-BODIPY® FL C_{11}-PC in 97 µL ethanol
- 100 µg Red/Green bis-BODIPY® PC-A2 in 101 µL DMSO

Store unused stock solutions at ≤–20ºC, desiccated, and protected from light.

1.2 Prepare 5 mM stock solutions of PED6 and PED-A1 by dissolving:

- 1 mg PED6 in 176 µL DMSO
- 100 µg PED-A1 in 22.7 µL DMSO

To prepare 1 mM stock solutions of PED6 and PED-A1 for use in fluorescence microplate assay protocol below, add 10 µL of 5 mM DMSO stock solution (prepared above) to 40 µL ethanol.

Store unused stock solutions at ≤–20ºC, desiccated, and protected from light.

1.3 Prepare a 10 mM stock solution of DOPC in ethanol. Store at –20°C.

1.4 Prepare a 10 mM stock solution of DOPG in ethanol. Store at –20°C.

Note: DOPG typically precipitates from ethanolic solution when stored at –20ºC but redissolves when warmed to room temperature. Anionic lipids such as DOPG generally enhance PLA_2 activity.

Experimental Protocol

Specific protocols for analysis of phospholipase A activity in nonadherent cells by flow cytometry and fluorescence microplate assays of enzyme preparations in solution are described below. Other protocols for biochemical and cell-based assays using fluorogenic phospholipase substrates may be adapted from published sources.[8–10]

Analysis of Nonadherent Cells by Flow Cytometry

The following protocol for incorporation of bis-BODIPY® FL C_{11}-PC into the plasma membrane inner leaflet via fusion with labeled liposomes is based on methods developed by Dr. Elizabeth Simons and colleagues.[11] This procedure is found to be effective with human neutrophils (PMNs) and other blood cell types such as platelets and lymphocytes.

2.1 Prepare a 2 mg/mL stock solution of PS in dry chloroform.

2.2 Mix approximately 12 µg bis-BODIPY® FL C_{11}-PC (12 µL of 1 mg/mL stock solution from step 1.1) with 88 µg PS (44 µL of 2 mg/mL stock solution from step 2.1). Evaporate the organic solvents under a stream of nitrogen or argon and place in a desiccated environment overnight.

2.3 Rehydrate the dried lipid film with 1 mL PBS and sonicate using a probe sonicator for 30 minutes on ice. This procedure results in the formation of small unilamellar liposomes, accompanied by a visible decrease in the turbidity of the sample. The molar composition ratio of the liposomes is 1:9 bis-BODIPY® FL C_{11}-PC:PS based on the above amounts of stock solution. Use the labeled liposomes within a few hours of preparation.

2.4 Mix labeled liposomes and a target cell suspension (for PMNs, a suspension of 6×10^7 cells/mL containing 0.1% bovine serum albumin was employed[11]). A mixture of 1 mL of liposomes with 0.5 mL cell suspension yields a preparation containing 2×10^7 cells and 8 µg bis-BODIPY® FL C_{11}-PC per mL.

2.5 Incubate the mixture at 37°C for about 1 hour, agitating slowly. Wash cells three times in PBS to remove any adherent liposomes. Store labeled cells in the dark at 4°C until further use.

2.6 Adjust the density of the loaded cell suspension to a level commensurate with instrumental sensitivity by diluting with a medium such as Krebs Ringer PBS (phosphate-buffered saline containing 1.5 mM Ca^{2+} and 1.5 mM Mg^{2+}, pH 7.3 ± 0.1). You may need to determine and optimize this level experimentally. A sample density of 2×10^6 PMNs per mL was employed for flow cytometric analysis.[11] This represents a 10-fold dilution of samples prepared according to step 2.4 and yields sufficient material for approximately 15 flow cytometric analyses.

Stimulus-induced phospholipase A activity results in a continuous increase of fluorescence.

Note: Addition of exogenous phospholipase A_2 (from *Crotalus adamanteus* venom, available from Sigma Chemical Co., St. Louis, MO) at 1 unit/mL elicits only a minimal fluorescence response in loaded cells, indicating localization of bis-BODIPY® FL C_{11}-PC in the inner leaflet of the plasma membrane.[11]

Fluorescence Microplate Assay for PLA_2

This protocol is suitable for analysis of secreted phospholipases A_2 and their inhibitors using bis-BODIPY® FL C_{11}-PC, Red/Green BODIPY® PC-A2, or PED6. The substrate is incorporated into liposomes prepared by injection of a concentrated solution of phospholipids in ethanol into the assay buffer[12].

3.1 Prepare 1 L assay buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM $CaCl_2$, pH 8.9) as follows:

Add the following reagents to 900 ml of deionized water.

Tris	6.057 g
NaCl	5.844 g
$CaCl_2.2H_2O$	0.147 g

Adjust the pH to 8.9 with HCl. Bring up the buffer volume to 1 L and mix. Store at 4°C.

3.2 In a conical microcentrifuge tube, mix 30 µL 10 mM DOPC (step 1.3), 30 µL 10 mM DOPG (step 1.4) and 30 µL 1 mM substrate in ethanol (step 1.1or 1.2).

3.3 Dispense 5 mL assay buffer (prepared in step 3.1) into a 20 mL beaker and stir rapidly with a small magnetic stir bar to form a vortex.

3.4 Slowly and steadily over about 1 minute, inject 77 µL of ethanolic lipid mix prepared in step 3.2 into the side of the vortex using a pipettor fitted with a narrow orifice gel-loading tip. This results in 5 mL of liposomally-incorporated substrate, sufficient for 100 assays in a 96-well plate format.

3.5 Pipette 50 µL samples of PLA_2-containing samples into microplate wells. Include at least one sample consisting of 50 µL assay buffer only, as a no-enzyme control.

Note: To generate a standard curve, prepare samples containing known enzyme concentrations in assay buffer. For group IB PLA_2 from bovine pancreas and group III PLA_2 from bee venom, a concentration range from 5 to 0.005 units/mL in a 2-fold serial dilution series is suitable. Note that the enzyme concentration is diluted 2-fold by addition of the substrate in step 3.7.

3.6 Set up the fluorescence microplate reader according to the specifications listed in Table 2. The assay gives a continuous kinetic readout. Set up a reading protocol according to individual kinetic sampling requirements and instrument capabilities. For initial survey experiments, we recommend reading all samples every 5 minutes for 1 hour, starting as soon as possible after addition of substrate (step 3.7).

3.7 Add 50 µL of substrate/DOPC/DOPG mixture (prepared in step 3.4) per sample well. This addition marks the zero time point for the kinetic assay. Commence fluorescence measurements as soon as possible after addition of the substrate.

References

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Cat. no.	Product Name	Unit Size
A10070	PED-A1 N-((6-(2,4-DNP)amino)hexanoyl)-1-(BODIPY®FL C_5)-2-hexyl-*sn*-glycero-3-phosphoethanolamine *phospholipase A_1 selective substrate*……..	100 µg
A10072	Red/Green BODIPY® PC-A2 (1-*O*-(6-BODIPY® 558/568-aminohexyl)-2-BODIPY® FL C_5-*sn*-glycero-3-phosphocholine) *ratiometric phospholipase A_2 substrate*…….	100 µg
B7701	1,2-bis-(4,4-difluoro- 5,7-dimethyl-4-bora-3a, 4a-diaza-*s*-indacene-3-undecanoyl)-*sn*-glycero-3-phosphocholine (bis-BODIPY® FL C_{11}-PC)…….	100 µg
D23739	*N*-((6-(2,4-dinitrophenyl) amino)hexanoyl)-2-(4,4- difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene- 3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (PED6)…….....	1 mg
D3834	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoic acid (BODIPY® FL C_5)……	1 mg
D3862	4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid (BODIPY® FL C_{11})…….....	1 mg

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