Fluorogenic Phospholipase A Substrates
Catalog nos. A10070, A10072, B7701, D23739

Table 1. Contents and storage information.

<table>
<thead>
<tr>
<th>Material</th>
<th>Amount</th>
<th>Storage</th>
<th>Stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>PED-A1 (A10070)</td>
<td>100 μg</td>
<td>≤–20ºC</td>
<td>When stored as directed the product is stable for at least 1 year.</td>
</tr>
<tr>
<td>N-((6-(2,4-DNP)amino)hexanoyl)-1-(BODIPY®FL C₅)-2-hexyl-sn-glycero-3-phosphoethanolamine</td>
<td></td>
<td>• Desiccate</td>
<td></td>
</tr>
<tr>
<td>Red/Green BODIPY® PC-A2 (A10072)</td>
<td>100 μg</td>
<td>• Protect from light</td>
<td></td>
</tr>
<tr>
<td>1-O-(6-BODIPY® 558/568-aminohexyl)-2-BODIPY® FL C₅-sn-glycero-3-phosphocholine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bis-BODIPY® FL C₁₁-PC (B7701)</td>
<td>100 μg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,2-bis-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoyl)-sn-glycero-3-phosphocholine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PED6 (D23739)</td>
<td>1 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>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</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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. 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. 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 PLA₁ versus PLA₂ 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 PLA₂-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 PLA₂ cleavage via their distinct spectroscopic signatures in imaging experiments.

Table 2. Fluorogenic phospholipase A substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cat. no.</th>
<th>Specificity</th>
<th>Structure</th>
<th>Cleavage Product*</th>
<th>Detection**</th>
</tr>
</thead>
<tbody>
<tr>
<td>bis-BODIPY® FL C₁₁-PC</td>
<td>B7701</td>
<td>PLA₁ or PLA₂</td>
<td>Figure 1 A</td>
<td>D3862</td>
<td>Fluorescence intensity increase, Ex = 488 nm, Em = 530 nm</td>
</tr>
<tr>
<td>Red/Green BODIPY® PC-A2</td>
<td>A10072</td>
<td>PLA₂</td>
<td>Figure 1 B</td>
<td>D3834</td>
<td>Fluorescence emission ratio increase, Ex = 488 nm, Em = 530/590 nm#</td>
</tr>
<tr>
<td>PED6</td>
<td>D23739</td>
<td>PLA₂***</td>
<td>Figure 2 A</td>
<td>D3834</td>
<td>Fluorescence intensity increase, Ex = 488 nm, Em = 530 nm</td>
</tr>
<tr>
<td>PED-A1</td>
<td>A10070</td>
<td>PLA₁</td>
<td>Figure 2 B</td>
<td>D3834</td>
<td>Fluorescence intensity increase, Ex = 488 nm, Em = 530 nm</td>
</tr>
</tbody>
</table>

*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 PLA₁ 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 C₁₁-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 PLA2. 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. PLA2 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. PLA2 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 PLA2 Fluorescence microplate assay**

- Phospholipase A2
  
  **Note:** This protocol was developed and tested using group IB PLA2 from bovine pancreas and group III PLA2 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 C11-PC and Red/Green BODIPY® PC-A2 by dissolving:
   - 100 μg bis-BODIPY® FL C11-PC in 97 μL ethanol
   - 100 μg Red/Green bis-BODIPY® PC-A2 in 101 μL DMSO
   
   Store unused stock solutions at ≤−20°C, 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 ≤−20°C, 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 −20°C but redissolves when warmed to room temperature. Anionic lipids such as DOPG generally enhance PLA2 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 C₁₁-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 C₁₁-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 C₁₁-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 × 10⁷ cells/mL containing 0.1% bovine serum albumin was employed¹¹). A mixture of 1 mL of liposomes with 0.5 mL cell suspension yields a preparation containing 2 × 10⁷ cells and 8 μg bis-BODIPY® FL C₁₁-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 Ca²⁺ and 1.5 mM Mg²⁺, pH 7.3 ± 0.1). You may need to determine and optimize this level experimentally. A sample density of 2 × 10⁶ 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 A₂ (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 C₁₁-PC in the inner leaflet of the plasma membrane.¹¹
Fluorogenic Phospholipase A Substrates

This protocol is suitable for analysis of secreted phospholipases A2 and their inhibitors using bis-BODIPY® FL C11-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.

3.1 Prepare 1 L assay buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM CaCl₂, pH 8.9) as follows:

Add the following reagents to 900 ml of deionized water.

- Tris: 6.057 g
- NaCl: 5.844 g
- CaCl₂·2H₂O: 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 1.1 or 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 PLA2-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 PLA2 from bovine pancreas and group III PLA2 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


Product List Current prices are available from www.invitrogen.com or from our Customer Service Department

<table>
<thead>
<tr>
<th>Cat. no.</th>
<th>Product Name</th>
<th>Unit Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>A10070</td>
<td>PED-A1 N-(6-(2,4-DNP)amino)hexanoyl)-1-(BODIPY®FL C₅)-2-hexyl-sn-glycero-3-phosphoethanolamine <em>phospholipase A₁ selective substrate</em>……………………..</td>
<td>100 μg</td>
</tr>
<tr>
<td>A10072</td>
<td>Red/Green BODIPY® PC-A2 (1-O-(6-BODIPY® 558/568-aminohexyl)-2-BODIPY® FL C₅-sn-glycero-3-phosphocholine) <em>ratiometric phospholipase A₂ substrate</em>…….</td>
<td>100 μg</td>
</tr>
<tr>
<td>B7701</td>
<td>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 C₁₁-PC)………………………..............</td>
<td>100 μg</td>
</tr>
<tr>
<td>D23739</td>
<td>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)...............................................................................</td>
<td>1 mg</td>
</tr>
<tr>
<td>D3834</td>
<td>4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoic acid (BODIPY® FL C₅)........................................................................................................</td>
<td>1 mg</td>
</tr>
<tr>
<td>D3862</td>
<td>4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY® FL C₁₁)......................................................................................................</td>
<td>1 mg</td>
</tr>
</tbody>
</table>
Further information on Molecular Probes products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East, contact our office in Paisley, United Kingdom. All others, contact our Technical Service Department in Eugene, Oregon.

Molecular Probes products are high-quality reagents and materials intended for research purposes only. These products must be used by, or directly under the supervision of, a technically qualified individual experienced in handling potentially hazardous chemicals. Please read the Material Safety Data Sheet provided for each product; other regulatory considerations may apply.

Limited Use Label License No. 223: Labeling and Detection Technology
The purchase of this product conveys to the buyer the non-transferable right to use the purchased amount of the product and components of the product in research conducted by the buyer (whether the buyer is an academic or for-profit entity). The buyer cannot sell or otherwise transfer (a) this product (b) its components or (c) materials made using this product or its components to a third party or otherwise use this product or its components or materials made using this product or its components for Commercial Purposes. The buyer may transfer information or materials made through the use of this product to a scientific collaborator, provided that such transfer is not for any Commercial Purpose, and that such collaborator agrees in writing (a) to not transfer such materials to any third party, and (b) to use such transferred materials and/or information solely for research and not for Commercial Purposes. Commercial Purposes means any activity by a party for consideration and may include, but is not limited to: (1) use of the product or its components in manufacturing; (2) use of the product or its components to provide a service, information, or data; (3) use of the product or its components for therapeutic, diagnostic or prophylactic purposes; or (4) resale of the product or its components, whether or not such product or its components are resold for use in research. If the purchaser is not willing to accept the limitations of this limited use statement, Invitrogen is willing to accept return of the product with a full refund. For information on purchasing a license to this product for purposes other than research, contact Molecular Probes, Inc., Business Development, 29851 Willow Creek Road, Eugene, OR 97402, Tel: (541) 465-8300. Fax: (541) 335-0354.

Several Molecular Probes products and product applications are covered by U.S. and foreign patents and patents pending. All names containing the designation ® are registered with the U.S. Patent and Trademark Office.

Copyright 2008, Molecular Probes, Inc. All rights reserved. This information is subject to change without notice.