Revised: 01–October–2004

Amplex® Red Phospholipase D Assay Kit (A12219)

Quick Facts

Storage upon receipt:
- −20°C
- Desiccate
- Protect from light

Abs/Em of reaction product: 571/585 nm

Introduction

The Amplex® Red Phospholipase D Assay Kit provides a sensitive method for measuring phospholipase D (PLD) activity *in vitro* using a fluorescence microplate reader or fluorometer. In this enzyme-coupled assay, PLD activity is monitored indirectly using 10-acetyl-3,7-dihydrophenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H_2O_2.[1,2] First, PLD cleaves the phosphatidylcholine (lecithin) substrate to yield choline and phosphatidic acid. Second, choline is oxidized by choline oxidase to betaine and H_2O_2. Finally, H_2O_2, in the presence of horseradish peroxidase, reacts with Amplex Red reagent in a 1:1 stoichiometry to generate the highly fluorescent product, resorufin.[1,3] Because resorufin has absorption and fluorescence emission maxima of approximately 571 nm and 585 nm, respectively (Figure 1), there is little interference from autofluorescence in most biological samples. The kit can be used to continuously assay PLD enzymes with near-neutral pH optima, whereas PLD enzymes with acidic pH optima can be assayed in a simple two-step procedure. Experiments with purified PLD from *Streptomyces chromofuscus* indicate that the Amplex Red PLD Assay Kit can detect PLD levels as low as 10 mU/mL using a reaction time of one hour. The kit is potentially useful for detecting PLD activity in cell extracts or for screening PLD inhibitors.

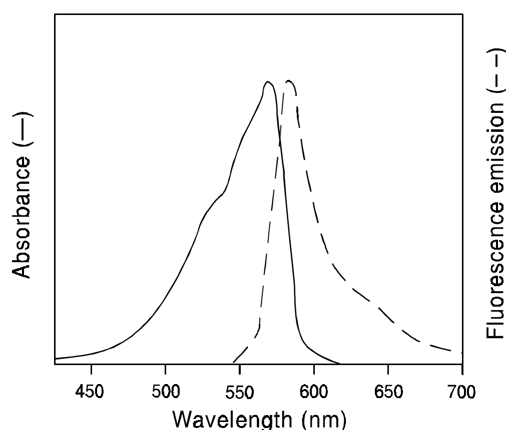

Figure 1. *Normalized absorption and fluorescence emission spectra of resorufin, the product of the Amplex Red reagent.*

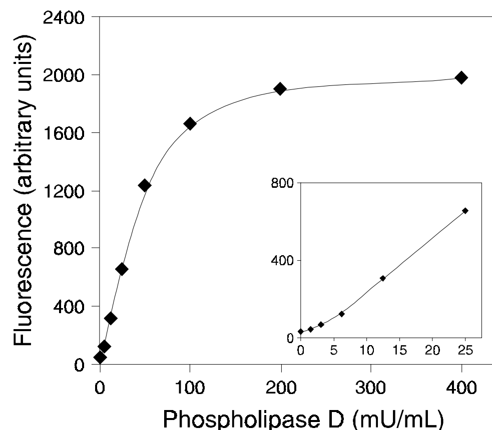

Figure 2. *Detection of PLD using the Amplex Red reagent–based assay. Each reaction contained 50 µM Amplex Red reagent, 1 U/mL HRP, 0.1 U/mL choline oxidase, 0.25 mM lecithin and the indicated amount of Streptomyces chromofuscus PLD in 1X Reaction Buffer. Reactions were incubated at 37°C for one hour. Fluorescence was measured with a fluorescence microplate reader using excitation at 530 ± 12.5 nm and fluorescence detection at 590 ± 17.5 nm.*

Materials

Kit Contents

- **Amplex Red reagent** (MW = 257, Component A), five vials, each containing 0.26 mg
- **Dimethylsulfoxide (DMSO)**, anhydrous (Component B), 0.7 mL
- **Horseradish peroxidase** (Component C), 200 U, where 1 unit is defined as the amount of enzyme that will form 1.0 mg purpurogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C
- **Hydrogen peroxide (H_2O_2)** (Component D), 500 µL of a stabilized ~3% solution; the actual concentration is indicated on the component label
- **5X Reaction Buffer** (Component E), 28 mL of 250 mM Tris-HCl, 25 mM $CaCl_2$, pH 8.0
- **Choline oxidase from *Alcaligenes* sp.** (Component F), 12 U, where 1 unit is defined as the amount of choline oxidase that will form 1.0 µmole of H_2O_2 due to oxidation of 1 µmole of choline to betaine aldehyde per minute at pH 8.0 at 37°C
- **L-α-Phosphatidylcholine (lecithin)** (MW ~760, Component G), 300 µL of a 100 mM solution in ethanol

Each kit provides sufficient reagents for approximately 500 assays using a fluorescence microplate reader and reaction volumes of 200 µL per assay.

Storage and Handling

Upon receipt, the kit should be stored frozen at -20°C, protected from light. Stored properly, the kit components should remain stable for at least six months. Allow reagents to warm to room temperature before opening vials. The Amplex Red reagent is somewhat air sensitive. Once a vial of Amplex Red reagent is opened, the reagent should be used promptly. PROTECT THE AMPLEX RED REAGENT FROM LIGHT.

Experimental Protocol

The following procedure is designed for use with a fluorescence multiwell plate scanner. For use with a standard fluorometer, volumes must be increased accordingly. Please note that the product of the Amplex Red reaction is unstable in the presence of thiols such as dithiothreitol (DTT) or 2-mercaptoethanol. For this reason, the final DTT or 2-mercaptoethanol concentration in the reaction should be less than 10 µM.

The absorption and fluorescence of resorufin are pH-dependent. Below the pK_a (~6.0), the absorption maximum shifts to ~480 nm and the fluorescence quantum yield is markedly lower. In addition, the Amplex Red reagent is unstable at high pH (>8.5). For these reasons, the reaction should be performed at pH 7–8. For assaying phospholipase D enzymes at moderately acidic pH, the reaction can be performed in two steps.

Stock Solution Preparation

1.1 Prepare a 10 mM stock solution of the Amplex Red reagent: Allow one vial of the Amplex Red reagent (Component A) and DMSO (Component B) to warm to room temperature. Just prior to use, dissolve the contents of the vial of Amplex Red reagent (0.26 mg) in 100 µL DMSO. Each vial of Amplex Red reagent is sufficient for approximately 100 assays, with a final reaction volume of 200 µL per assay. This stock solution should be stored frozen at -20°C, protected from light.

1.2 Prepare a 1X working solution of Reaction Buffer by adding 5 mL of 5X Reaction Buffer stock solution (Component E) to 20 mL of deionized water (dH_2O). This 25 mL volume of 1X Reaction Buffer is sufficient for approximately 100 assays of 200 µL each, with a 5 mL excess for making stock solutions and dilutions.

1.3 Prepare a 200 U/mL stock solution of horseradish peroxidase (HRP) by dissolving the contents of the vial of HRP (Component C) in 1.0 mL of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at -20°C.

1.4 Prepare a 20 mM H_2O_2 working solution by diluting the ~3% H_2O_2 stock solution (Component D) into the appropriate volume of dH_2O. The actual H_2O_2 concentration is indicated on the component label. For instance, a 20 mM H_2O_2 working solution can be prepared from a 3.0% H_2O_2 stock solution by diluting 23 µL of 3.0% H_2O_2 into 977 µL of dH_2O. Please note that although the ~3% H_2O_2 stock solution has been stabilized to slow degradation, the 20 mM H_2O_2 working solution will be less stable and should be used promptly.

1.5 Prepare a 20 U/mL stock solution of choline oxidase by dissolving the contents of the vial of choline oxidase (Component F) in 600 µL of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at -20°C.

1.6 *Optional:* If desired as a positive control, prepare a stock solution of PLD (not provided) in 1X Reaction Buffer. We recommend using PLD from *Streptomyces chromofuscus* (Sigma, catalog number P 8023; see Figure 2).

Continuous PLD Assay (for PLD Enzymes with Near-Neutral pH Optima)

The following protocol describes the assay of PLD in a total volume of 200 µL per microplate well. The volumes recommended here are sufficient for ~100 assays.

2.1 Dilute the PLD–containing samples in 1X Reaction Buffer. Use 1X Reaction Buffer without PLD as a negative control. A volume of 100 µL will be used for each reaction.

2.2 Prepare a positive control by diluting the 20 mM H_2O_2 working solution to 10 µM in 1X Reaction Buffer.

2.3 *Optional:* Prepare another positive control by diluting the PLD stock solution (prepared in step 1.6) into 1X Reaction Buffer.

2.4 Pipet 100 µL of the diluted samples and controls into separate wells of a microplate.

2.5 Prepare a working solution of 100 µM Amplex Red reagent containing 2 U/mL HRP, 0.2 U/mL choline oxidase and 0.5 mM lecithin by adding 100 µL of Amplex Red reagent stock solution (prepared in step 1.1), 100 µL of HRP stock solution (prepared in step 1.3), 100 µL of choline oxidase stock solution (prepared in step 1.5) and 50 µL of the lecithin solution (Component G) to 9.65 mL of 1X Reaction Buffer. Note that this solution may be milky in appearance due to the lecithin. This 10 mL volume is sufficient for ~100 assays. Final concentrations of each component will be twofold lower in the final reaction volume.

2.6 Begin the reactions by adding 100 µL of the Amplex Red reagent/HRP/choline oxidase/lecithin working solution to each microplate well containing the samples and controls.

2.7 Incubate the reactions for 30 minutes or longer at 37°C, protected from light. Because the assay is continuous (not terminated), fluorescence may be measured at multiple time points to follow the kinetics of the reactions.

2.8 Measure the fluorescence in a fluorescence microplate reader using excitation in the range of 530–560 nm and emission detection at ~590 nm (see Figure 1).

2.9 For each point, correct for background fluorescence by subtracting the values derived from the no-PLD control.

Two-Step PLD Assay (for PLD Enzymes with Acidic pH Optima)

Some PLD enzymes have acidic pH optima. To assay these enzymes, you may wish to perform a two-step assay in which the PLD reaction is performed at a lower pH, and then the pH is

raised to allow detection with the Amplex Red reagent. The following protocol can be used as a guideline for performing a two-step assay. The volumes recommended here are sufficient for ~100 assays, using a final reaction volume of 200 µL per assay.

3.1 Dilute the PLD–containing samples in the reaction buffer of your choice. Use reaction buffer without PLD as a negative control. A volume of 100 µL will be used for each reaction.

3.2 Add 0.5 µL of the 100 mM lecithin solution (Component G) to each sample or negative control.

3.3 Incubate the first-step reactions at 37°C for the desired length of time (e.g., one hour).

3.4 Prepare a positive control by diluting the 20 mM H_2O_2 working solution to 10 µM in 1X Reaction Buffer.

3.5 *Optional:* While the reactions are incubating, prepare another positive control by diluting the PLD stock solution (prepared in step 1.6) into 1X Reaction Buffer.

3.6 Pipet 100 µL of the diluted controls into separate wells of a microplate.

3.7 Add 0.5 µL of the 100 mM lecithin solution (Component G) to each control.

3.8 Prepare a working solution of 100 µM Amplex Red reagent containing 2 U/mL HRP and 0.2 U/mL choline oxidase by adding 100 µL of Amplex Red reagent stock solution (prepared in step 1.1), 100 µL of HRP stock solution (prepared in step 1.3) and 100 µL of choline oxidase stock solution (prepared in step 1.5) to 9.7 mL of 1X Reaction Buffer. This 10 mL volume is sufficient for ~100 assays. Concentrations of each component will be twofold lower in the final reaction volume.

3.9 Begin the second step reactions by adding 100 µL of the Amplex Red reagent/HRP/choline oxidase working solution to each microplate well containing the samples and controls.

3.10 Incubate the reactions for 30 minutes or longer at 37°C, protected from light.

3.11 Measure the fluorescence in a fluorescence microplate reader using excitation in the range of 530–560 nm and emission detection at ~590 nm (see Figure 1).

3.12 For each point, correct for background fluorescence by subtracting the values derived from the no-PLD control.

References

1. Anal Biochem 253, 162 (1997); **2.** Proc SPIE-Int Soc Opt Eng 3926, 166 (2000); **3.** J Immunol Methods 202, 133 (1997).

Product List

Current prices may be obtained from our Web site or from our Customer Service Department.

Cat #	Product Name	Unit Size
A12219	Amplex® Red Phospholipase D Assay Kit *500 assays*	1 kit
A12222	Amplex® Red reagent (10-acetyl-3,7-dihydroxyphenoxazine)	5 mg
A22177	Amplex® Red reagent *packaged for high-throughput screening*	10 x 10 mg
A36006	Amplex® UltraRed reagent	5 x 1 mg

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