

Amplex[®] Red Phosphatidylcholine-Specific Phospholipase C Assay Kit (A12218)

Quick Facts

Storage upon receipt:

- -20°C
- Desiccate
- Protect from light

Abs/Em of reaction product: 571/585 nm

Introduction

The Amplex[®] Red Phosphatidylcholine-Specific Phospholipase C Assay Kit provides a sensitive method for continuously monitoring phosphatidylcholine-specific phospholipase C (PC-PLC) activity *in vitro* using a fluorescence microplate reader or fluorometer. In this enzyme-coupled assay, PC-PLC activity is monitored indirectly using 10-acetyl-3,7-dihydrophenoxazine (Amplex Red reagent), a sensitive fluorogenic probe for H₂O₂.¹ First, PC-PLC converts the phosphatidylcholine (lecithin) substrate to form phosphocholine and diacylglycerol. After the action of alkaline phosphatase, which hydrolyzes phosphocholine, choline is oxidized by choline oxidase to betaine and H₂O₂.² Finally, H₂O₂, 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

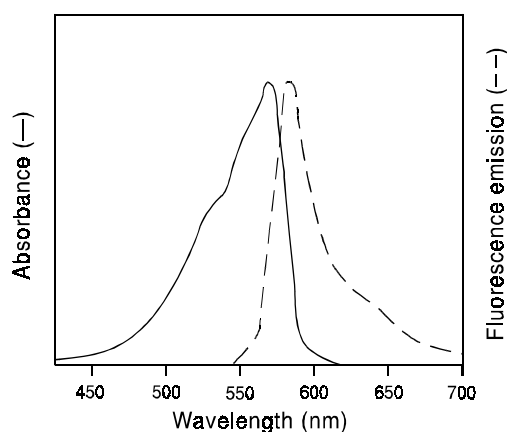


Figure 1. Normalized absorption and fluorescence emission spectra of resorufin, the product of the Amplex Red reagent.

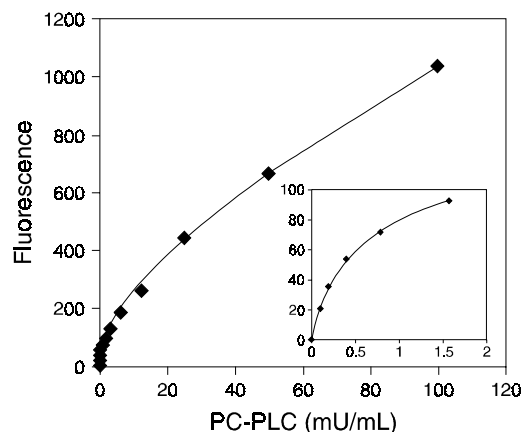


Figure 2. Detection of PC-PLC using the Amplex Red reagent-based assay. Each reaction contained 200 μM Amplex Red reagent, 1 U/mL HRP, 4 U/mL alkaline phosphatase, 0.1 U/mL choline oxidase, 0.5 mM lecithin and the indicated amount of *B. cereus* PC-PLC in 1X Reaction Buffer. Reactions were incubated at 37°C for one hour. Fluorescence was measured with a fluorescence microplate reader using excitation at 560 ± 10 nm and fluorescence detection at 590 ± 10 nm.

interference from autofluorescence in most biological samples. Experiments with purified PC-PLC from *Bacillus cereus* indicate that the Amplex Red PC-PLC Assay Kit can detect PC-PLC levels as low as 0.2 mU/mL using a reaction time of one hour (Figure 2). The kit is potentially useful for detecting PC-PLC activity in cell extracts or for screening PC-PLC inhibitors.

Materials

Kit Contents

- **Amplex Red reagent** (MW = 257, Component A), five vials, each containing 1 mg
- **Dimethylsulfoxide (DMSO)**, anhydrous (Component B), 1.3 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 (H₂O₂)** (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, pH 7.4, 0.70 M NaCl, 50 mM dimethylglutarate, 10 mM CaCl₂

- **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
- **Alkaline phosphatase from calf intestine** (Component G), 500 U, where 1 unit is defined as the amount of enzyme that will hydrolyze 1 μmole of 4-nitrophenyl phosphate per minute in 1 M diethanolamine buffer, pH 9.8, at 37°C
- **Phosphatidylcholine (lecithin)** (MW ~775, Component H), 600 μL of a 100 mg/mL (~129 mM) solution in ethanol
- **Phosphatidylcholine-specific phospholipase C from *Bacillus cereus*** (Component I), 2 U, where 1 unit is defined as the amount of enzyme that will liberate 1.0 μmole of water-soluble organic phosphorus from L- α -phosphatidylcholine per minute at pH 7.3 at 37°C.

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. We recommend using the included Reaction Buffer (pH 7.4) for optimal performance of the Amplex Red reagent.

Stock Solution Preparation

1.1 Prepare an ~20 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 (1 mg) in 200 μL DMSO. Each vial of Amplex Red reagent is sufficient for approximately 100 assays of 200 μL each. 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 Prepare a 400 U/mL stock solution of alkaline phosphatase by dissolving the contents of the vial of alkaline phosphatase (Component G) in 1.25 mL of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at -20°C.

1.7 Prepare a 10 U/mL stock solution of *B. cereus* PC-PLC by dissolving the contents of the vial of PC-PLC (Component I) in 200 μL of 1X Reaction Buffer. After use, the remaining solution should be divided into small aliquots and stored frozen at -20°C.

PC-PLC Assay

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

2.1 Dilute the PC-PLC-containing samples in 1X Reaction Buffer. A volume of 100 μL will be used for each reaction.

2.2 Prepare a positive control by diluting the 10 U/mL PC-PLC stock solution (prepared in step 1.7) into 1X Reaction Buffer to produce a 0.1 U/mL PC-PLC solution. Use 1X Reaction Buffer without PC-PLC as a negative control. A volume of 100 μL will be used for each reaction.

2.3 Prepare another positive control by diluting the 20 mM H_2O_2 working solution to 10 μM in 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 400 μM Amplex Red reagent containing 2 U/mL HRP, 8 U/mL alkaline phosphatase, 0.2 U/mL choline oxidase and 1 mM lecithin by adding 200 μL of Amplex Red reagent stock solution (prepared in step 1.1), 100 μL of HRP stock solution (prepared in step 1.3), 200 μL of alkaline phosphatase stock solution (prepared in step 1.6), 100 μL of choline oxidase stock solution (prepared in step 1.5) and 78 μL of the lecithin solution (Component H) to 9.32 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/alkaline phosphatase/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 termi-

nated), 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-PC-PLC 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 |
|--------|---|------------|
| A12218 | Amplex [®] Red Phosphatidylcholine-Specific Phospholipase C 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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