

EnzChek® Direct Phospholipase C Assay Kit

Phosphatidylcholine-specific

Catalog nos. E10215, E10216

Table 1. Contents and storage information.

Material	E10215 (2-plates)	E10216 (10-plates)	Concentration	Storage*	Stability
Phospholipase C substrate (Component A)	2 vials	10 vials	NA	<ul style="list-style-type: none"> • ≤−20°C • Desiccate • Protect from light 	When stored as directed, the product is stable for at least 1 year.
Dimethylsulfoxide (DMSO) (Component B)	200 µL	1 mL		Desiccate	
5X Phospholipase C reaction buffer (Component C)	12 mL	60 mL	250 mM Tris-HCl, pH 7.4, 0.7 M NaCl, 10 mM CaCl ₂	<ul style="list-style-type: none"> • ≤6°C* • Desiccate 	
Phosphatidylcholine (lecithin) (Component D)	100 µL	500 µL	100 mM in ethanol	≤−20°C	
Phospholipase C from <i>Bacillus cereus</i> , phosphatidylcholine-specific (PC-PLC) (Component E)	8 Units	40 Units	NA	<ul style="list-style-type: none"> • ≤−20°C • Desiccate 	

*For short-term storage (1–2 weeks), the buffer (Component C) may be stored at room temperature; however, for long-term storage we recommend storage at ≤6°C to prevent microbial contamination. NA = not applicable.

Number of assays: For Cat. no. E10215, sufficient material is supplied for 200 reactions in 96-well microplates at a volume of 200 µL per well as described in the protocol below, or 2,000 reactions using low-volume 384-well microplates at a volume of 20 µL per well. For Cat. no. E10216, sufficient material is supplied for 1,000 reactions in 96-well microplates at 200 µL per well as described in the protocol below, or 10,000 reactions using low-volume 384-well microplates at a volume of 20 µL per well.

Approximate fluorescence excitation/emission maxima: 509/516 in nm.

Introduction

The EnzChek® Direct Phospholipase C Assay Kit provides a simple and robust method for monitoring phosphatidylcholine-specific phospholipase C (PC-PLC) activity. The PC-PLC enzyme plays a crucial role in many cell signaling pathways involved in apoptosis and cell survival, as well as diseases ranging from cancer to HIV.¹⁻⁷ The assay uses a proprietary substrate (glycero-phosphoethanolamine with a dye-labeled *sn*-2 acyl chain) to detect PC-PLC activity. Substrate cleavage by PC-PLC releases the dye-labeled diacylglycerol, which produces a positive fluorescence signal that can be measured continuously using a fluorescence microplate reader. The reaction product has fluorescence excitation and emission maxima of 509 nm and 516 nm, respectively (Figure 1). The assay has been

optimized using purified PC-PLC from *Bacillus cereus*. The assay may be amenable for use with cells and cell lysates, although the presence of phospholipase A2 and/or D enzymes can potentially result in signal enhancement. Using purified enzyme from *Bacillus cereus*, the assay can detect as little as 10 mU/mL PC-PLC after one hour incubation at room temperature (Figure 2). The kit is useful for characterizing PC-PLC inhibition (Figure 3), and since it offers a direct measurement, the potential for false positives in a compound screen is eliminated.

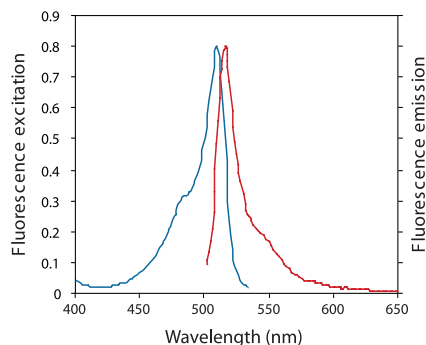


Figure 1. Normalized excitation and emission spectra for PLC substrate after reaction with PC-PLC. Samples were incubated in the presence of 2 U/mL PC-PLC from *B. cereus* for approximately 60 minutes at room temperature.

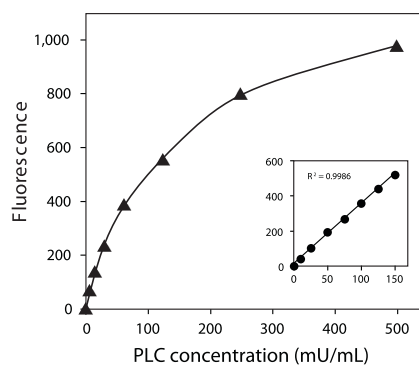


Figure 2. Detection of PC-PLC using the EnzChek® Direct PLC Assay Kit. Triplicate samples from *B. cereus* were assayed at concentration of 7.8 mU/mL to 500 mU/mL per well in the presence of 1X PLC substrate and 200 μ M lecithin in 1X PLC reaction buffer. Reactions were incubated at room temperature for 60 minutes and fluorescence was measured at Ex/Em 490/520 nm. The inset represents a separate experiment and illustrates the linearity of fluorescence response at low levels of PC-PLC. The average variation of replicates (CV) was less than 3%. Background fluorescence has been subtracted.

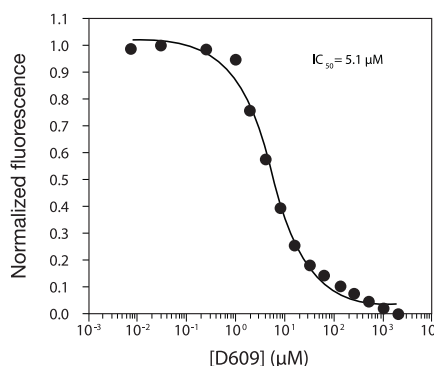


Figure 3. IC_{50} determination for D609, a known competitive PC-PLC inhibitor⁸⁻¹¹ with a K_i of 6.4 μ M¹², using the EnzChek® Direct PLC Assay Kit. Triplicate samples of D609 were titrated in the presence of 1X PLC substrate, 200 μ M lecithin and, 100 mU/mL PC-PLC in 1X PLC reaction buffer. Reactions were incubated for 60 minutes at room temperature and fluorescence was measured at Ex/Em 490/520 nm using a fluorescent microplate reader.

Before You Begin

Materials Required but Not Provided

- Samples
- Deionized water
- Plastic vials for reagent preparation
- Microplates, 96-well or 384-well

General Guidelines

- The kit is useful for detecting PC-PLC activity in samples or screening PC-PLC inhibitors.
- The assay protocol below is optimized for use with 96-well microplates using a 200 μL reaction volume per assay. For 384-well plates, adjust the reaction volumes accordingly to 20 μL per assay (recommended).
- The assay protocol is designed for use with a fluorescence microplate reader. A SpectraMax M5 (Molecular Devices) was used throughout the development of this kit.
- The assay performs best using black/clear-bottom microplates, although various types of microplates have been tested with the assay, including 96-well clear and 96-well black/clear bottom, standard 384-well black/clear bottom, and low-volume 384-well white, using top and bottom read modes, where applicable. All plates produce similar results, although sensitivity and dynamic range may be affected (Figure 4).
- Allow the kit components to equilibrate to room temperature before use.
- Use the included PLC reaction buffer for optimal performance.

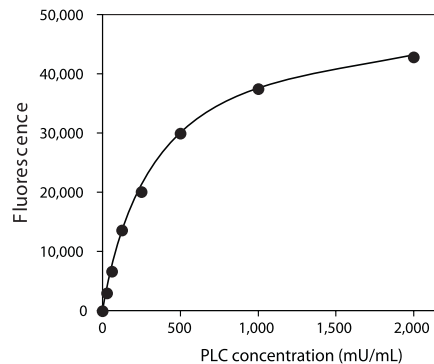


Figure 4. Sensitivity and dynamic range of the EnzChek® Direct PLC Assay Kit using low-volume 384-well white microplates. Triplicate PC-PLC samples from *B. cereus* were assayed at concentrations of 31.25 mU/mL to 2,000 mU/mL per well in the presence of 1X PLC substrate and 200 μM lecithin in 1X reaction buffer. Reactions were incubated at room temperature for 60 minutes and fluorescence was measured at Ex/Em 490/520 nm. The average variation of replicates (CV) was less than 5%. Background fluorescence has been subtracted.

Preparing Solutions

- 1.1 Dilute the 5X PLC reaction buffer (Component C) 5-fold with deionized water.

For example, add 5 mL 5X PLC reaction buffer to 20 mL deionized water. This volume of 1X PLC reaction buffer is sufficient for 100 assays of 200 μ L each, with a 5 mL excess for making stock solutions and dilutions.

- 1.2 Prepare a 200X stock solution of PLC substrate (Component A). Add 100 μ L DMSO (Component B) to one vial of PLC substrate. This volume is sufficient for 100 assays of 200 μ L each. Store any remaining stock solution at room temperature, **protected from light**.

Note: Substrate stock solution in DMSO appears yellow-orange. If no color is visible, the substrate is too dilute.

- 1.3 Prepare a 40 U/mL stock solution of purified PC-PLC from *Bacillus cereus* (Component E) as follows:

For 2-plate kit (Cat. no. E10215), dissolve the contents of Component E in 200 μ L of 1X PLC reaction buffer (prepared in step 1.1).

For 10-plate kit (Cat. no. E10216) dissolve the contents of Component E in 1 mL of 1X PLC reaction buffer (prepared in step 1.1).

After use, aliquot the remaining solution into small aliquots and store at $\leq -20^{\circ}\text{C}$. Sufficient enzyme is included to prepare 80 (Cat. no. E10215) or 400 (Cat. no. E10216) positive control samples at 500 mU/mL in an assay volume of 200 μ L. You can use other sources of PC-PLC as a positive control, but the sensitivity and dynamic range of the assay may be affected.

Experimental Protocol

Perform the standard assay protocol below using a total volume of 200 μ L per well. Mix the samples and controls with the substrate working solution at a ratio of 1:1 (100 μ L sample/control + 100 μ L substrate), such that the concentration of each component is two-fold lower in the final reaction volume. You may use other volumes if you maintain the ratio of samples/controls to substrate at 1:1.

Assay Protocol

- 2.1 Prepare a 1 U/mL PC-PLC positive control by diluting the 40 U/mL stock solution (prepared in step 1.3) 40-fold in 1X PLC reaction buffer.

For example, add 25 μ L of 40 U/mL PC-PLC positive control stock solution to 0.975 mL 1X PLC reaction buffer.

- 2.2 Prepare a serial dilution of PC-PLC directly in the wells of a microplate to obtain an 8-point standard curve of PC-PLC concentrations of 0–1 U/mL, each in a volume of 100 μ L. Final PC-PLC concentration is two-fold lower (0–0.5 U/mL).

For example, aliquot 200 μ L 1 U/mL PC-PLC positive control in 1X PLC reaction buffer to well A1 of the microplate. Aliquot 100 μ L of 1X PLC reaction buffer to the remaining wells of column A and prepare 2-fold serial dilutions down the plate by removing 100 μ L PC-PLC from the top well (A1), adding this to the well below (B1), mixing and repeating. Leave the last well (H1) as a buffer-only negative control, and discard 100 μ L from the previous well so that each well contains 100 μ L of PC-PLC solution.

- 2.3 Dilute your PC-PLC containing samples appropriately in 1X PLC reaction buffer and aliquot 100 μ L samples appropriately in the plate containing positive control.
- 2.4 Prepare a working solution of PLC substrate by adding 40 μ L lecithin (Component D) and 100 μ L of PLC substrate stock solution (prepared in step 1.2) to 9.86 mL 1X PLC reaction buffer. Mix well. Note that this solution may appear milky due to the presence of lecithin.
- 2.5 Add 100 μ L substrate working solution (from step 2.4) to the wells containing PC-PLC samples and controls. Mix well.
- 2.6 Cover the microplate and incubate for 30 minutes or longer (up to overnight) at room temperature, **protected from light**. Because the assay is continuous (not terminated), you can measure fluorescence at multiple time points to follow the reaction kinetics, if desired.
- 2.7 Measure the fluorescence in a microplate reader. Wavelength settings of 490/520 nm also work well in the assay.
- 2.8 Use a standard curve to determine the sample (unknown) PC-PLC concentrations by plotting the enzyme concentration for the positive controls against fluorescence and fitting a line to the data points.

Inhibitor Screening

For screening PC-PLC inhibitors, select the concentration of enzyme which yields a signal increase at least 20% of maximal.

For IC₅₀ determinations, prepare serial dilutions of the inhibitor(s) of interest and include the selected amount of PC-PLC in the substrate working solution at 2X the final concentration.

References

1. Toxicol. 44, 161 (2004); 2. Dev Neurosci. 28, 499 (2006); 3. Cancer Sci. 99, 696 (2008); 4. Blood 111, 3355 (2008); 5. Antioxid Redox. Signal 7, 1217 (2005); 6. Journal of Leukocyte Biology 80, 407(2006); 7. Eur J Immunol. 37, 2912 (2007); 8. Cell Signal 10, 321 (1998); 9. Proc Natl Acad Sci USA. 101, 9745 (2004); 10. Eur J Immunol. 36, 277 (2006); 11. Exp Cell Res. 299, 370 (2004); 12. Drugs Exp Clin Res. 22, 287 (1996).

Troubleshooting

Problem	Cause	Solution
No response from the control enzyme	Low substrate concentration or substrate is contaminated	Substrate stock solution in DMSO appears yellow-orange. If no color is visible, the substrate is too dilute. If color is visible, but there is no response, the substrate is contaminated. Repeat experiment with fresh substrate.
No response from samples	PC-PLC absent, inactivated, or is present in low quantities	Increase incubation time or enzyme amount. If no signal, repeat the experiment with a fresh vial of substrate.
Response not in the linear range	PC-PLC in the sample is highly active or sample contains PLA ₂ , or PLD enzyme, or both	Dilute sample until response falls within linear range of the standard curve. Ensure samples are free from phospholipase A ₂ (PLA ₂) or phospholipase D (PLD) enzymes.

Product List Current prices may be obtained from our website or from our Customer Service Department.

Cat. no.	Product Name	Unit Size
E10215	EnzChek® Direct Phospholipase C Assay Kit *phosphatidylcholine specific* *2 Plates*	1 kit
E10216	EnzChek® Direct Phospholipase C Assay Kit *phosphatidylcholine specific* *10 Plates*	1 kit

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