Total Phosphatidic Acid Fluorometric Assay Kit

Item No. 700240

Customer Service 800.364.9897 * **Technical Support** 888.526.5351
www.caymanchem.com

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GENERAL INFORMATION

Materials Supplied

Kit will arrive packaged as a -20°C kit. For best results, remove components and store as stated below.

Item Number	Item	Quantity	Storage
700241	PA Assay Buffer (10X)	1 vial	-20°C
700242	PA Assay Lipase	2 vials	-20°C
700243	Phosphatidic Acid Standard	1 vial	-20°C
700244	PA Assay Enzyme Mixture	3 vials	-20°C
700245	PA Assay ADHP	3 vials	-20°C
700001	DMSO Assay Reagent	1 vial	-20°C
700247	1% Triton X-100	1 vial	Room temperature
400091	Half Volume 96-Well Plate (black)	1 plate	Room temperature
400012	96-Well Cover Sheet	1 cover	Room temperature

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: This product is for laboratory research use only: not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Precautions

Please read these instructions carefully before beginning this assay.

For research use only. Not for human or diagnostic use.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Fax: 734-971-3641

Email: techserv@caymanchem.com

Hours: M-F 8:00 AM to 5:30 PM EST

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A fluorometer with the capacity to measure fluorescence using an excitation wavelength between 530-540 nm and an emission wavelength between 585-595 nm
2. Methanol, chloroform, and 1 M sodium chloride solution to be used for extracting cellular lipids (see **Sample Preparation** on page 9)
3. Adjustable pipettes and a multichannel or repeating pipettor
4. A source of pure water; glass distilled water or HPLC-grade water is acceptable
5. Protein Determination Kit (Cayman Item No. 704002) for standardizing Phosphatidic Acid (PA) concentration to milligram protein-*optional*

INTRODUCTION

Background

Phosphatidic acid (PA) is the acid form of phosphatidate, a common phospholipid that is a major constituent of cell membranes and a central intermediate for the synthesis of membrane lipids and storage lipids.[1] Besides *de novo* synthesis, PA can be formed in three ways: 1) phospholipase D (PLD) hydrolysis of the phosphodiester bond of phosphatidylcholine (PC) to produce PA and free choline; 2) phosphorylation of diacylglycerol (DAG) by DAG kinase (DAGK); and 3) acylation of lysophosphatidic acid (LPA) by lyso-PA-acyltransferase (LPAAT).[1-4] PA can be metabolized to DAG by lipid phosphate phosphohydrolases (LPPs) or to LPA by phospholipase A (PLA).[1]

PA is linked to many intracellular signal transduction events.[2] PA has been identified as a critical component of mammalian target of rapamycin (mTOR) signaling that regulates both cell cycle progression and cell growth.[5] The disregulation of the mTOR pathway is implicated as a contributing factor to various human diseases, especially various types of cancer.[6,7] Rapamycin is a bacterial natural product that can inhibit mTOR through association with its intracellular receptor FKBP12. PA directly interacts with mTOR at the domain targeted by rapamycin and enzymes that generate PA are potential regulators of mTOR.[8] Raf-1 kinase, sphingosine kinase 1, and cAMP phosphodiesterase 4A1 contain PA binding sites and require direct interaction with PA for translocation.[2-4] PA also modulates the membrane localization and/or activity of protein kinase C, the tyrosine phosphatase SHP-1, PLC, and the guanine nucleotide exchange factor Son of sevenless.[2-4]

About This Assay

Cayman's Total PA Fluorometric Assay provides a fluorescence-based method for measuring total PA (PA and LPA) in cellular lipids. Lipase is used to hydrolyze PA, as well as any LPA that is present, to glycerol-3-phosphate, which is then oxidized by glycerol-3-phosphate oxidase to generate hydrogen peroxide (H_2O_2). In the presence of peroxidase, H_2O_2 reacts with ADHP (10-acetyl-3,7-dihydroxyphenoxazine) to yield the highly fluorescent compound resorufin (see Figure 1, on page 7).[9] Resorufin fluorescence can be analyzed using excitation wavelengths of 530-540 nm and emission wavelengths of 585-595 nm.

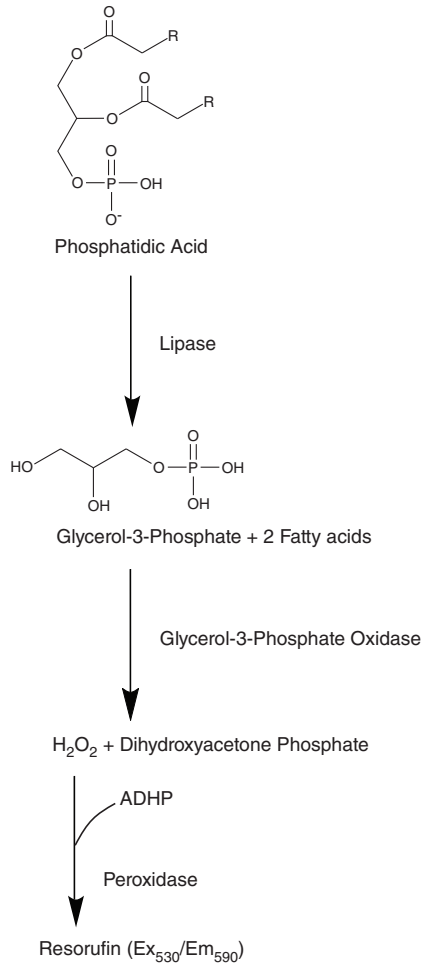

Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. PA Assay Buffer (10X) - (Item No. 700241)

The vial contains 10 ml of 500 mM Tris-HCl, pH 7.4, containing 500 mM sodium chloride. Dilute 5 ml of Assay Buffer with 45 ml of HPLC-grade water. This final Assay Buffer (50 mM Tris-HCl, pH 7.4, containing 50 mM sodium chloride) will be used in the assay and for diluting reagents. When stored at 4°C, this diluted Assay Buffer is stable for at least six months.

2. PA Assay Lipase - (Item No. 700242)

Each vial contains 2.2 ml of lipase. Thaw and store the enzyme on ice while preparing the reagents for the assay. One vial will assay 55 wells. The enzyme is ready to use as supplied. Store the unused Lipase at -20°C.

3. Phosphatidic Acid Standard - (Item No. 700243)

The vial contains 1 ml of a 625 µM solution of phosphatidic acid (PA) from egg yolk lecithin. The reagent is ready to use to prepare the PA standard curve. Store unused standard at -20°C.

4. PA Assay Enzyme Mixture - (Item No. 700244)

Each vial contains lyophilized glycerol-3-phosphate oxidase and horseradish peroxidase. It will be used to make the Detector Mixture (see page 14 for instructions). One vial will make enough Detector Mixture to assay 40 wells.

5. PA Assay ADHP - (Item No. 700245)

Each vial contains a clear lyophilized powder of 10-acetyl-3,7-dihydroxyphenoxazine (ADHP). It will be used to make the Detector Mixture (see page 14 for instructions). Once it is reconstituted, ADHP is stable for 15 minutes. After 15 minutes, increased background fluorescence will occur.

6. DMSO Assay Reagent - (Item No. 700001)

The vial contains 1 ml of dimethylsulfoxide (DMSO). The reagent is ready to use as supplied. Once thawed, DMSO may be stored at room temperature.

7. 1% Triton X-100 - (Item No. 700247)

The vial contains 25 ml of 1% Triton X-100. The reagent is ready to use as supplied.

Sample Preparation

This kit is designed for detection of PA in cellular lipids, not in serum or plasma samples. Extract lipids using a modified method of Bligh and Dyer as outlined on page 10.[10,11] Make the Preequilibrated Upper Phase (PEU) that will be used to wash the chloroform phase by mixing 50 ml of chloroform, 50 ml of methanol, and 45 ml of 1 M sodium chloride in a glass bottle or beaker with a stir bar. Shake or mix the solution and then allow it to separate into two phases. Use the upper phase for the washes as described on page 10.

Extracting Cellular Lipids

NOTE: 15 or 50 ml conical polypropylene centrifuge tubes can be used to extract the lipids.

1. Collect cells (~8 x 10^6) by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, harvest using a rubber policeman; Do not use proteolytic enzymes.
2. Pour off the media and suspend the cell pellet in 0.5-1 ml of cold 1X PBS, pH 7.4, or buffer of choice.
3. Sonicate the cell pellet on ice.
4. We recommend doing a protein determination on the sample to normalize the PA concentration to protein concentration (nmol PA/mg protein). The protein determination has to be done before extracting the lipids. A Protein Determination Kit (Item No. 704002) can be purchased from Cayman.
5. After performing a protein determination, add 1.5 ml of methanol to the sonicated pellet.
6. Add 2.25 ml of 1 M sodium chloride and 2.5 ml of chloroform to the sample and then vortex.
7. Centrifuge the sample for 10 minutes at 1,500 x g at 4°C to separate the phases.
8. Remove the upper aqueous phase and discard.
9. Wash the lower chloroform phase twice with 2 ml preequilibrated upper phase (PEU). Separate phases after each wash by centrifuging at 1,500 x g for 10 minutes at 4°C. Remove and discard the upper phase after separation.
10. After the second PEU wash, remove the upper phase and transfer the lower chloroform phase (a syringe works well) to a 12 x 75-mm glass tube, being careful not to transfer the remnant upper phase.
11. Dry the lower phase under a gentle stream of nitrogen and resuspend the sample in 500 µl of 1% Triton X-100 (Item No. 700247). If not assaying the same day, freeze at -80°C. The extracted sample will be stable for at least one month while stored at -80°C. Samples can be diluted with Assay Buffer before performing the assay.

ASSAY PROTOCOL

Plate Set Up

There is no specific pattern for using the wells on the plate. However, a PA standard curve in duplicate has to be assayed with the samples. We suggest that each sample be assayed at least in duplicate (triplicate recommended) and to record the contents of each well on the template sheet provided on page 23. A typical layout of standards and samples to be measured in duplicate is given below.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A	A	S2	S2	S10	S10	S18	S18	S26	S26	S34	S34
B	B	B	S3	S3	S11	S11	S19	S19	S27	S27	S35	S35
C	C	C	S4	S4	S12	S12	S20	S20	S28	S28	S36	S36
D	D	D	S5	S5	S13	S13	S21	S21	S29	S29	S37	S37
E	E	E	S6	S6	S14	S14	S22	S22	S30	S30	S38	S38
F	F	F	S7	S7	S15	S15	S23	S23	S31	S31	S39	S39
G	G	G	S8	S8	S16	S16	S24	S24	S32	S32	S40	S40
H	S1	S1	S9	S9	S17	S17	S25	S25	S33	S33	S41	S41

A-G = Standards
S1-S41 = Sample Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps to maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 100 µl in all the wells.
- All reagents except the enzymes must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- We recommend assaying samples in triplicate, but it is the user's discretion to do so.
- The assay is performed at 37°C.
- Monitor the fluorescence on a fluorometer with an excitation wavelength in the range of 530-540 nm and an emission wavelength of 585-595 nm.

Standard Preparation

Take seven clean glass test tubes and mark them A-G. Add the amount of PA Standard and Assay Buffer to each tube as described in Table 1. The diluted standards are stable for four hours at room temperature.

Tube	PA Standard (µl)	Assay Buffer (µl)	Final Concentration (µM)
A	0	500	0
B	10	490	12.5
C	20	480	25
D	40	460	50
E	60	440	75
F	80	420	100
G	100	400	125

Table 1. PA standards to be assayed along with samples

Performing the Assay

1. **Standard Wells** - add 10 µl of standard (tubes A-G) per well in the designated wells on the plate (see **Sample Plate Format**, Figure 2, page 11).
2. **Sample Wells** - add 10 µl of sample to at least two wells. To obtain reproducible results, the amount of PA added to the wells should fall within the range of the assay. When necessary, samples should be diluted with Assay Buffer.
3. Initiate the reactions by adding 40 µl of Lipase to every well being used.
4. Cover the plate with the plate cover and incubate at 37°C for one hour.
5. Prepare the **Detector Mixture** within 15 minutes prior to adding to the wells: Add 1.8 ml of diluted Assay Buffer to the PA Assay Enzyme Mixture (Item No. 700244) vial and vortex. Add 100 µl of DMSO Assay Reagent (Item No. 700246) to the PA Assay ADHP vial (Item No. 700245), vortex, add 400 µl of diluted Assay Buffer, and vortex. To the reconstituted Enzyme Mixture, add 200 µl of resuspended ADHP and vortex. This is enough Detector Mixture for 40 wells. Prepare additional Detector Mixture as needed. The Detector Mixture is stable for 15 minutes.
6. Remove plate cover, add 50 µl of Detector Mixture to all of the wells being used, cover, and incubate for 30 minutes at room temperature.
7. Remove the plate cover and read the plate using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm. You may need to adjust the instrument GAIN to detect the entire range of the standards.

ANALYSIS

Calculations

1. Determine the average fluorescence of each standard and sample. Subtract the fluorescence value of standard A from itself and all other standards and samples. This is the corrected fluorescence.
2. Plot the corrected fluorescence values of each standard as a function of the final concentration of PA from Table 1. See Figure 3, on page 17, for a typical standard curve.
3. Calculate the PA concentration of the samples using the equation obtained from the linear regression of the standard curve substituting the corrected sample fluorescence (CSF) for each sample.

$$\textbf{Phosphatidic acid concentration (µM)} = \left[\frac{\text{CSF - (y-intercept)}}{\text{slope}}\right] \text{ x Sample Dilution}$$

4. Standardize the PA concentration in each sample to protein concentration by dividing the PA concentration by protein concentration.

$$\textbf{nmol PA/mg protein} = \left[\frac{\text{µM}}{\text{mg/ml}}\right]$$

Performance Characteristics

Precision:

When a series of sixteen HEK293 lipid extract measurements were performed on the same day, the intra-assay coefficient of variation was 3.2%. When a series of sixteen HEK293 lipid extract measurements were performed on six different days under the same experimental conditions, the inter-assay coefficient of variation was 4.2%.

Assay Range:

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0-125 µM of PA.

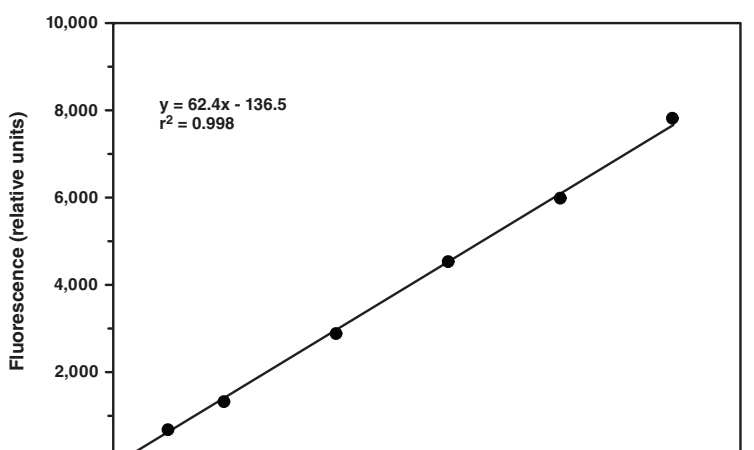

Figure 3. PA standard curve

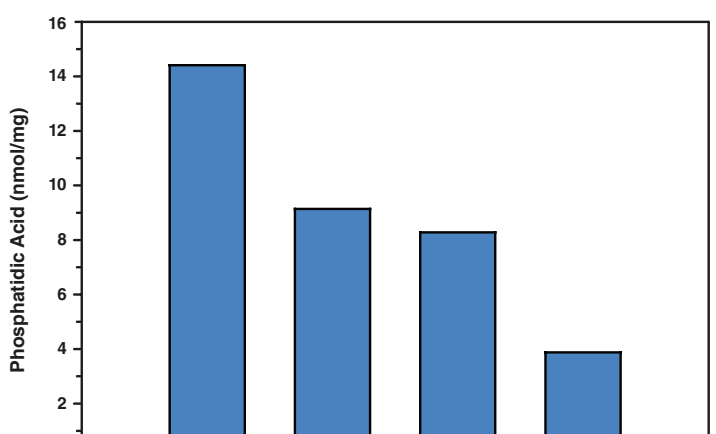

Figure 4. PA content in HEK293 lipid extracts

HEK293 cells were grown on 10-cm dishes in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) in a humidified incubator (5% CO_2) at 37°C until 100% confluency. HEK293 cells were then treated with either vehicle (DMSO, control), 50 µM CAY10594 (PLD inhibitor), 50 µM R59949 (DAGK inhibitor), or with 50 µM of both inhibitors in DMEM containing 10% FBS at 37°C for 24 hours. After the treatment, the cellular lipids were extracted according to the procedure outlined in **Sample Preparation**. The PLD and DAGK inhibitors markedly reduced the cellular PA content, confirming that both enzymes are involved in PA production in HEK293 cells.[8,9]

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence detected above background in sample wells	Sample was too dilute	Re-assay the sample using a lower dilution
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	Reduce the GAIN and re-read
Fluorescence in the sample wells are above the last standard of the PA standard curve	Sample is too concentrated	Dilute the sample with assay buffer and re-assay; Assaying a few dilutions of each sample will help avoid this problem

References

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Related Products

Autotaxin Inhibitor Screening Assay Kit - Item No. 700580
Phosphatidylcholine Colorimetric Assay Kit - Item No. 10009926
Phospholipase D Assay Kit - Item No. 700590
Protein Determination Kit - Item No. 704002
Sphingomyelin Colorimetric Assay Kit - Item No. 10009928
Triglyceride Colorimetric Assay Kit - Item No. 10010303

Warranty and Limitation of Remedy

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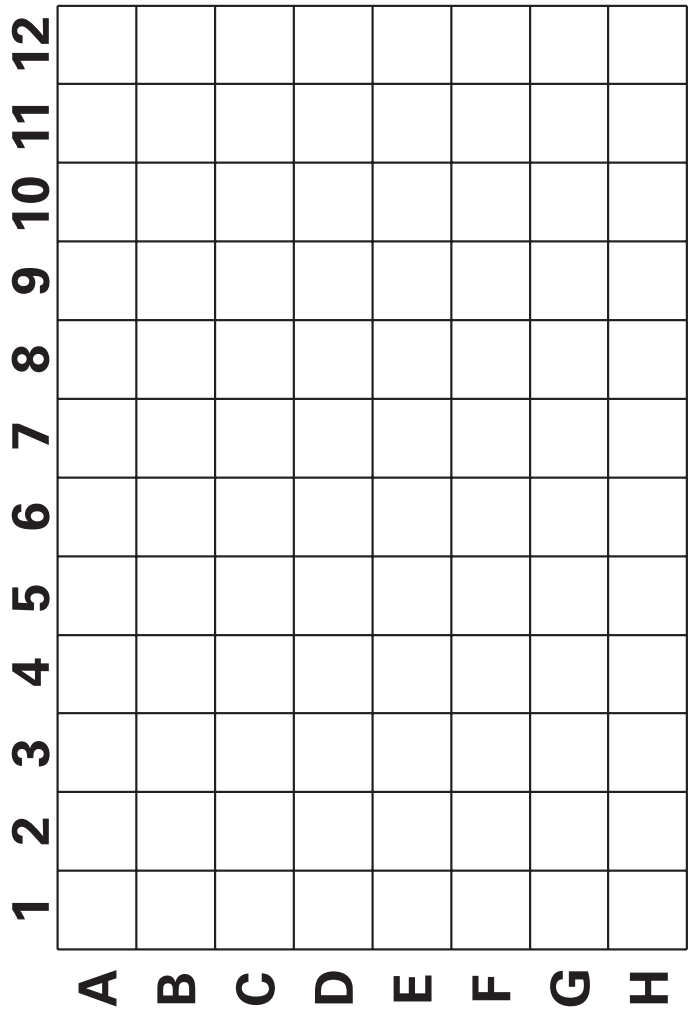
Buyer's **exclusive remedy** and Cayman's sole liability hereunder shall be limited to a refund of the purchase price, or at Cayman's option, the replacement, at no cost to Buyer, of all material that does not meet our specifications.

Said refund or replacement is conditioned on Buyer giving written notice to Cayman within thirty (30) days after arrival of the material at its destination. Failure of Buyer to give said notice within thirty (30) days shall constitute a waiver by Buyer of all claims hereunder with respect to said material.

For further details, please refer to our Warranty and Limitation of Remedy located on our website and in our catalog.



NOTES

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