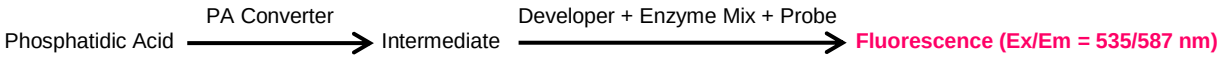
PicoProbe™ Phosphatidic Acid Assay Kit (Fluorometric)

(Catalog # K748-100; 100 assays; Store at -20°C)

I. Introduction:

Phospholipids are amphiphilic molecules containing phosphate groups, and are both critical components of biological membranes and important signaling messengers in a number of biological pathways. Phosphatidic acid (PA) is the simplest phospholipid, consisting of a glycerol backbone with fatty acids bonded to carbon 1 and 2, and a phosphate group bonded to carbon 3. PA is a precursor for the synthesis of more complex phospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine that act as structural components of the plasma membrane. Phosphatidic acid itself is present in the plasma membrane lending the membrane a high curvature and a net negative charge. In addition, it is an important signaling lipid. It acts through a G-protein coupled receptor and is involved in growth regulation and mitosis. BioVision's phosphatidic acid assay kit is a plate-based enzymatic assay for quantitation of PA in cells and tissues. The PA Converter hydrolyzes PA to form an intermediate which, in the presence of a developer and enzyme mix, converts a non-fluorescent probe to a fluorescent product (Ex/Em= 535/587 nm) that can be quantified. The Converter is specific to PA and does not hydrolyze other phospholipids (e.g. those with more complex head groups such as phosphatidylcholine or cardiolipin), allowing direct quantitation of PA. This kit can detect as low as 40 pmol PA per well.



II. Applications:

- Measurement of phosphatidic acid content in lipid extracts from cells and tissues

III. Sample Type:

- Cell lipid extract
- Tissue lipid extract

IV. Kit Contents:

Components	K748-100	Cap Code	Part Number
PA Assay Buffer	100 ml	WM	K748-100-1
PA Converter	1 vial	Orange	K748-100-2
PA Developer	1 vial	Green	K748-100-3
PA Enzyme Mix	1 vial	Red	K748-100-4
PicoProbe™ (in DMSO)	400 µl	Blue	K748-100-5
PA Standard (1 mM)	100 µl	Yellow	K748-100-6

V. User Supplied Reagents and Equipment:

- 96-well flat bottom clear plate
- Multi-well spectrophotometer
- Water bath / heating plate / oven
- Chloroform
- Methanol
- 12 N HCl
- 1 M NaCl
- Triton X-100
- 10 ml conical vials
- Glass test tubes (5 ml / 10 ml)

VI. Storage Conditions and Reagent Preparation:

Upon arrival, store the kit at -20°C, protected from light. Centrifuge vials prior to opening. Read the protocol before performing the assay.

- **PA Assay Buffer:** Warm to room temperature before use.
- **PA Converter, PA Developer and PA Enzyme Mix:** Store at -20°C. Lyophilized vials are stable for at least 6 months. Reconstitute PA Converter in 880 µl PA assay buffer, and PA Developer and Enzyme in 220 µl assay buffer each before use. Aliquot remaining components and store at -20°C. Reconstituted vials are stable for at least two months.
- PicoProbe™**:** Store at -20°C. Thaw at room temperature before use. Aliquot and store at -20°C.
- **PA Standard:** Store at -20°C. Thaw in a water bath at 37°C for 15-20 minutes. Aliquot and store at -20°C.

VII. Phosphatidic Acid Assay Protocol:

1. **Sample Preparation:** ***Sample preparation may take several hours / overnight. Complete sample preparation before preparing other reagents for the assay. Chloroform that is used for sample preparation is toxic, therefore sample preparation should be done under a fume hood.*** Homogenize tissue (~100 mg; non-perfused) or cells (~1 million) in 1 ml PA assay buffer. Protein content of the sample may be determined at this stage if desired. We recommend *BCA protein assay kit (BV# K813-2500)*. Carry out lipid extraction according to the following protocol: add 3.75 ml of chloroform/methanol/12N HCl (2 : 4 : 0.1 v/v). Mix thoroughly and add 1.25 ml of chloroform to 1 ml of cell/tissue homogenate. Vortex for 30 seconds and add 1.25 ml of 1 M NaCl. Mix well and centrifuge at 3000 x *g* for 10 minutes at room temperature. *Two distinct layers will be visible at this point*. Collect the lower organic layer (containing solubilized lipids) with a pipette and transfer to a glass tube. Allow the chloroform to evaporate in a vacuum oven or a regular oven

(37°C) overnight or until the chloroform evaporates completely. Extracted lipids will stick to the walls of the tube and should be solubilized in 50 - 500 µl 5% Triton X-100 solution. If not being used immediately, the lipid extract may be stored at -80°C for 3-4 days. Add 2 to 10 µl of samples into wells of a 96-well clear plate. For each sample prepare two wells; "Sample background control" and "Sample". Bring the volume of "Sample" wells to 50 µl and "Sample background control" wells to 70 µl with PA Assay buffer.

Note: Different dilutions of sample should be tested to make sure that Phosphatidic acid concentration falls in the linear range of the assay. Samples should be diluted using PA Buffer.

2. **Phosphatidic acid Standard Curve Generation:** Dilute the provided 1 mM PA standard 1:10 in PA Assay Buffer to obtain 100 µM PA. Add 0, 2, 4, 6, 8, and 10 µl of the 100 µM PA standard to wells of the 96 well plate to obtain 0, 0.2, 0.4, 0.6, 0.8 and 1 nmol of Phosphatidic acid per well. Bring up the total volume in these wells to 50 µl with PA Assay buffer.

3. **Converter Mix:** Mix enough reagents for the number of assays to be performed. For each sample and standard well, prepare 20 µl:

	Converter Mix
PA Assay Buffer	12 µl
PA Converter	8 µl

Add 20 µl the converter mix to wells containing the samples and standards. Mix well. *Do not add the converter mix to "Sample background control" wells*. Incubate at 45 °C for 1 hour**.**

4. **Reaction mix:** Mix enough reagents for the number of assays to be performed. For each well, prepare 30 µl. The total reaction volume after addition of reaction mix is 100 µl.

	Reaction Mix
PA Assay Buffer	22 µl
PA Developer	2 µl
PA Enzyme mix	2 µl
PA Probe	4 µl

Add 30 µl of the reaction mix to all wells. Mix well. Incubate at 37°C for 30 minutes**.**

5. **Measurement:** Record fluorescence in end point mode at Ex/Em= 535/587 nm.

6. **Calculations:** Subtract 0 PA reading from all PA standard readings. Plot the Phosphatidic acid Standard Curve. Subtract sample background control readings from sample readings. If 0 PA readings are higher than sample background control readings, subtract those form sample readings instead. Apply corrected RFU to Standard Curve to get B nmol PA in the sample well.

PA concentration in sample (C) = (B / V) X D (nmol/ml) or µM

Where **B** = Amount of Phosphatidic acid in the sample well from Standard Curve (nmol)
V = Volume of sample added into the well (ml)
D = Dilution factor

PA molecular weight: 711 g/mol
PA concentrations can also be expressed as nmol PA per mg protein or nmol PA per mg tissue weight.

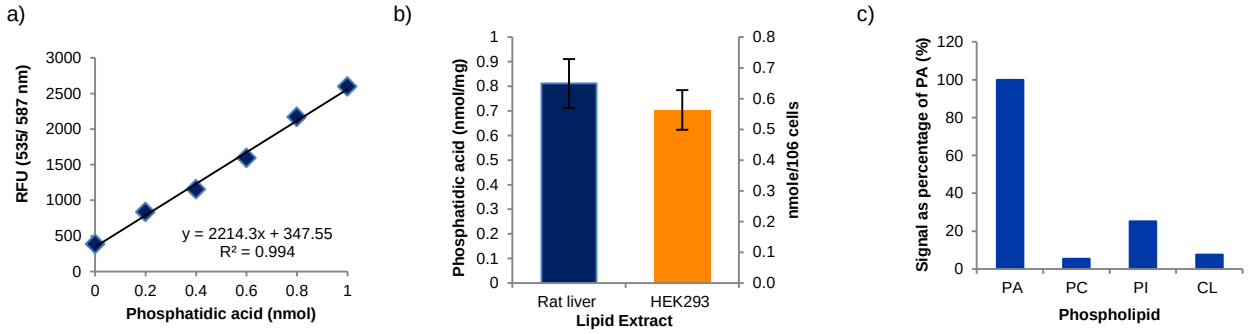


Figure: (a) Phosphatidic acid standard curve. (b) Phosphatidic acid content in rat liver and HEK 293 cells. For rat liver extract, 100 mg tissue was homogenized in 1 ml PA assay buffer. Lipid extraction was carried out as described above and lipid extract was re-suspended in 200 µl 5% Triton X-100 solution. For HEK293, cells were grown to confluency and harvested using a rubber policeman. Extract was prepared as described above. (c) Assay Specificity: Equimolar amounts (20 nmol) of phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylinositol (PI) and cardiolipin (CL) show interference less than 20% with respect of Phosphatidic Acid. Assay shows specificity for phosphatidic acid. Experiments were performed using kit protocol.

VIII. RELATED PRODUCTS

Phospholipid Assay Kit (K351)
Cardiolipin Assay Kit (K944)
Phosphatidylcholine Colorimetric/Fluorometric Assay Kit (K576)
Triglyceride Assay Kit (K622)
Sphingomyelin Quantification Colorimetric Assay Kit (K600)
Glycerol-3-Phosphate (G3P) Colorimetric Assay Kit (K641)
Phospholipase D (PLD) Activity Colorimetric Assay Kit (K725)
Free Glycerol Colorimetric Assay Kit II (K634)
Glycerol Cell-Based Assay Kit (K977)
Phosphatidylethanolamine Assay Kit (K499)

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