Echelon Biosciences Inc.

PI(4)P Mass ELISA Assay Kit

K-4000E (96 tests)

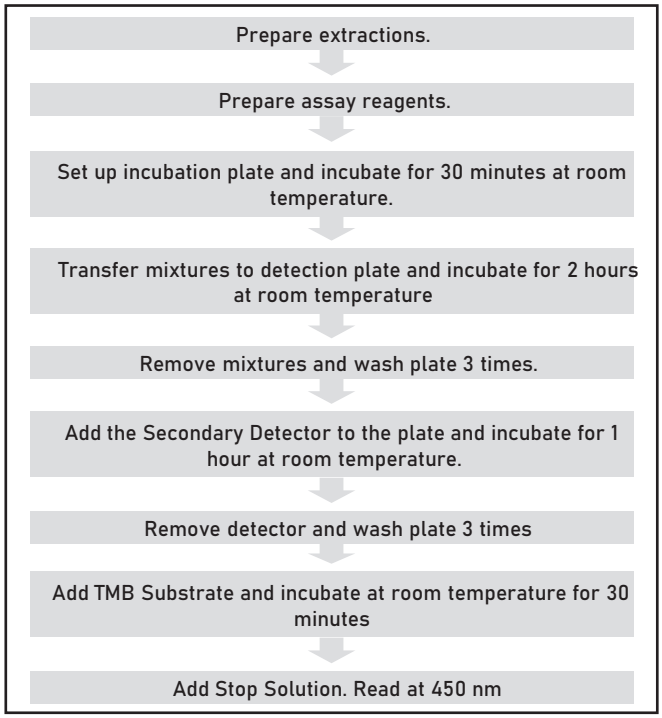
Support: echelon@echelon-inc.com

Description: 96-well ELISA assay for the detection and quantification of PI(4)P from

Materials Provided

Catalog #	Description	Amount
K-4005	PI(4)P Detector	1 vial
K-4001E	PI(4)P Detection Plate	1 plate
K-4006	PI(4)P (diC16) Standard	1 vial
K-DIL2	Diluent	lyophilized pellet
K-PBST2	10X PBS-T Buffer	20 mL
K-SEC2	Secondary Detector	300 µL
K-TMB1	TMB Substrate	12 mL
K-STOPt	1 N H_2SO_4 Stop Solution	10 mL
---	Colored 96-well polypropylene U-bottom plate	1 plate
---	Clear acetate sheet, 1 side adhesive	3

Quick Protocol



Additional Materials Provided by User

- Extracted PI(4)P samples (See Support Protocol PI(4)P extraction at the end of this document)
- Buffers and solvents for PI(4)P extractions: Trichloroacetic Acid, EDTA, Methanol, Chloroform, and 12 N HCl
- Absorbance microtiter plate reader capable of reading at 450 nm.
- Vacuum dryer

Storage The kit comes in two parts with different storage requirements. Upon receipt store Kit Part 1 at 4°C and Kit Part 2 at -80°C. Store prepared reagents as indicated in the protocol.

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Background

Phosphatidylinositol 4-phosphate (PtdIns(4)P, PI(4)P) is the most abundant monophosphorylated phosphoinositide found in mammalian cells[1]. It is produced by PtdIns 4-Kinases (PI4K) which phosphorylate the D-4 position of the inositol ring of PtdIns. PI(4)P is then converted to PtdIns(4,5)P2 by PtdIns(4)P 5-Kinases.

Assay Design

Echelon's PI(4)P Mass ELISA kit is designed to detect and quantify PI(4)P by means of a competitive ELISA format, eliminating the need for radioactivity and thin layer chromatography. The PI(4)P Mass ELISA directly detects PI(4)P over all other phosphionositides at relevant biological levels.

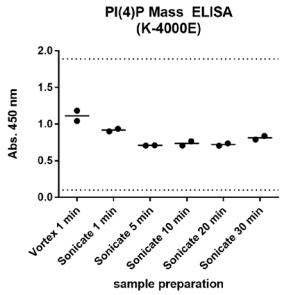
The PI(4)P Mass assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(4)P measured. The PI(4)P extractions are added to the colored mixing plate with PI(4)P Detector. This mixture is then transferred to the PI(4)P Detection Plate for competitive binding. A peroxidase-linked secondary detector and colorimetric detection is used to detect the amount of PI(4)P Detector binding to the plate. The assay is sensitive to 0.7 pmol PI(4)P.

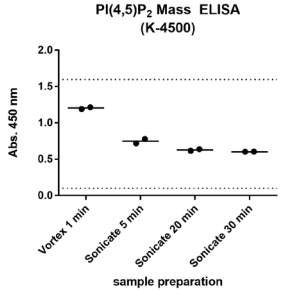
Disclaimer

The PIP Mass Assays are used to quantify the total amount of the specific lipid extracted from cells. However, since the lipids are substrates for enzymes in multiple pathways the data obtained may not correlate with what has been observed with isolated enzyme reactions or visualized with immunohistochemistry.

Assay Notes

1. The incubation steps for this assay require a plate shaker. If a plate shaker is unavailable the incubation steps can be run for two hours without shaking. A reduction in signal and some loss in sensitivity may be observed.
2. The PI(4)P Detection Plate is composed of 12 8-well strips. Unused strips should be removed from the plate frame and stored in a clean sealable plastic bag at 4°C. Save the plate frame after assay for future use of the remaining strip wells.
3. Never let the detection plate dry out after the ELISA assay has started. Always prepare the next solution needed before discarding the current one from wells in use.
4. For lipid extraction questions please see "PIP Mass FAQ: PIP Mass Customer Information Sheet" located on the webpage for this product. This document can also be requested at echelon@echelon-inc.com.
5. It can be difficult to reproduce conditions of sonication; due to variation in the number of vials between batches, temperature of the water bath, and sonicator tuning. The suggested sonication time of 5-10 min was developed using a water bath sonicator. The results observed with your water bath sonicator may be differ





ent. It is suggested that your sonicator is tested with PI(4)P extraction samples for day to day variation and time dependent consistency. If you are running other lipid mass assays you may want to consider dissolving your lipids the same manner. How you dissolve the lipid will affect how it goes into solution and can cause inconstancies in your data if it is not held constant.

Protocol for the detection of PI(4)P

Please read the entire Assay Procedure, Lipid Extraction Protocol, and Assay Notes before beginning assay. Bring PI(4)P Standard (K-4006), PI(4)P extraction samples, Buffers (K-PBST2, K-DIL2), TMB Solution (K-TMB1), 1 N H2SO4 Stop Solution (K-STOPt), and Detection Plate (K-4001E) to room temperature before use. Place PI(4)P Detector (K-4005) and Secondary Detector (K-SEC2) on ice until use.

Buffer Preparation

1. PBS-T Buffer Prepare by adding the entire bottle of 10X PBS-T Buffer (K-PBST2) to 180 mL DI water. Mix. Keep at room temperature.

2. Working Diluent Prepare by adding 12 mL DI water to the bottle of Diluent (K-DIL2). Vortex. Let sit 5 minutes. Vortex. Keep at room temperature until use. For long term storage keep Working Diluent at -20°C.

PI(4)P Mass ELISA General Protocol

1. Prepare PI(4)P extraction samples and PI(4)P Standards
   a. Make sure PI(4)P Standard (K-4006), PI(4)P extraction samples, and PBS-T buffer are at room temperature before proceeding to next step.
   b. Add 558 µL of PBS-T Buffer to the vial of PI(4)P standard (K-4006). Vortex 1 min at moderate speed to resuspend lipid. Follow with centrifugation. This is the 450 pmol/well standard. Further dilute 450 pmol/well standard in 0.5 mL tubes, using the dilution table below. The 450 pmol/well standard can be stored at -20°C for 1 month and can withstand one freeze/defrost cycle.

PI(4)P Standard curve dilution table			
Row	pmol/well	Vol. stock solution or previous dilution	Vol. PBS-T
B	450.00	200 µL 450 pmol/well Standard	0 µL
C	150.00	100 µL 450 pmol/well Standard	200 µL
D	50.00	100 µL previous dilution	200 µL
E	16.66	100 µL previous dilution	200 µL
F	5.55	100 µL previous dilution	200 µL
G	1.85	100 µL previous dilution	200 µL

   c. To the PI(4)P extraction samples add 200 µL PBS-T Buffer then sonicate 5-10 min in a room temperature water bath. Do not add ice to water bath. See assay notes section for more information on sample preparation.

2. Prepare working PI(4)P Detector (K-4005) in Working Diluent
   a. Add 50 µL of dH2O to the vial of PI(4)P Detector (K-4005) for a 10 µg/mL stock. Keep on ice for 1 minute to fully dissolve protein. Mix vial by flicking with finger. Do not vortex. The 10 µg/mL PI(4)P Detector stock solution can be stored up to 3 months at -20°C.
   b. Further dilute the PI(4)P Detector by adding 40 µL of the 10 µg/mL stock (prepared in step 2a) to 7 mL Working Diluent. Keep at room temperature and proceed immediately to next step.
3. Set up Colored Mixing Plate using plate layout as a guide

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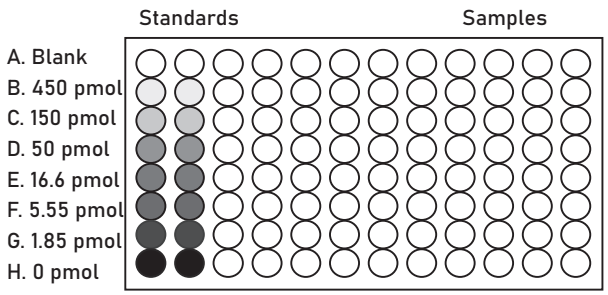
a. Add 60 µL/well prepared PI(4)P Standards (step 1b) to standard wells.
b. Add 60 µL/well prepared PI(4)P extraction samples (step 1c) to extraction sample wells.
c. Add 60 µL/well PBS-T Buffer to Blank and 0 pmol wells.
d. Add 60 µL/well prepared 0.05 µg/mL PI(4)P Detector (step 2b) to all wells except Blank control.
e. Add 60 µL/well working Diluent to Blank control.



4. Seal plate with plate sealer and incubate on plate shaker for 30 minutes at room temperature.

5. Transfer from Colored Mixing Plate to PI(4)P Detection Plate.
   a. From each well of the Colored Mixing Plate transfer 100 µ L to the corresponding well of the PI(4)P Detection Plate (K-4001E). For best results, mix each well 3-6 times with pipette before transferring.
   b. Seal plate with plate sealer and incubate on plate shaker for 2 hours at room temperature.

6. Discard protein/lipid solution and wash PI(4)P Detection Plate 3 times with 200 µL/well PBS-T Buffer. Leave last wash in plate and proceed to next step

7. Prepare and add Secondary Detector to PI(4)P Detection Plate.
   a. Add 200 µL of Secondary Detector (K-SEC2) and 3 mL Diluent to 9 mL PBS-T Buffer. Mix well.
   b. Discard PBS-T Buffer from PI(4)P Detection Plate and add 100 µL/well prepared Secondary Detector (step 7a) to all wells of PI(4)P Detection Plate.
   c. Seal plate with plate sealer and incubate on plate shaker for 1 hour at room temperature.

8. Discard Secondary Detector and wash PI(4)P Detection Plate 3 times with 200 µL/well PBS-T Buffer. Remove last wash from plate and proceed to next step.

9. Develop plate
   a. Add 100 µL/well TMB (K-TMB1) to PI(4)P Detection Plate and develop for 30 minutes at room temperature in a dark location.
   b. Stop reaction by adding 50 µL/well 1 N H_2SO_4 Stop Solution (K-STOPt) and read at 450 nm with an absorbance plate reader.

Data Analysis

Generate a best fit curve for the PI(4)P standards and interpolate relative sample values (See figure 1. for example). The final PI(4)P concentration, for each sample, should include any dilutions made to the sample before it is added to the Colored Mixing Plate.

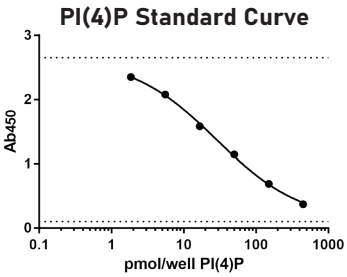


Figure 1.
PI(4)P standard curve was generated using non-linear regression analysis with GraphPad software. A log(agonist) vs. response -- Variable slope (four parameters) analysis was utilized. For best results, constrain standard top and bottom using the 0 pmol PI(4)P and Blank control.

Support Protocol: Lipid Extraction

The PI(4)P extraction protocol was verified with 2 x 10^6 HL-60 cells. The amount of cells necessary for PI(4)P quantification needs to be determined for each cell type. Larger or smaller amounts of cells require proportional adjustments of volumes. If you have never run lipid extractions or have little experience with the reagents listed below, please read the "PIP Mass FAQ: PIP Mass Customer Information Sheet" before running extractions. The FAQ can be found on the webpage of this product or it can be requested at echelon@echelon-inc.com.

Solutions for Extraction

1. 0.5 M TCA For 50 mL, dissolve 4.08 g TCA (Trichloroacetic Acid) in dH2O and bring volume to 50 mL.
2. 5% TCA with 1 mM EDTA For 50 mL, dissolve 2.5 g TCA in dH2O, add 100 µL 0.5 M EDTA, and bring volume to 50 mL with dH2O.
3. MeOH:CHCl3 (2:1) For 60 mL, add 40 mL MeOH to 20 mL CHCl3
   a. Measure CHCl3 with a glass pipette. Pure CHCl3 may dissolve plasticware.
   b. MeOH: CHCl3 (2:1) should be prepared in an amber glass bottle. This solution is not stable long term and should be used within a month of preparation. It's safe to use plasticware to transfer this solution.
4. MeOH:CHCl3:HCl (80:40:1): For 60 mL, combine 40 mL MeOH, 20 mL CHCl3, and 0.5 mL 12 N HCl
   a. Measure CHCl3 with a glass pipette. Pure CHCl3 may dissolve plasticware.
   b. MeOH:CHCl3:HCl (80:40:1) should be prepared in an amber glass bottle. This solution is not stable long term and should be used within a month of preparation. It's safe to use plasticware to transfer this solution.
   c. Use 12 N concentrated 36% - 38% HCl. Do not use diluted acid.
5. 0.1 N HCl For 50 mL, add 0.42 mL 12 N HCl to 50 mL dH2O.

Extraction of PI(4)P from cells

1. Collect Cells
   a. For adherent cells in a 25 cm2 flask, 35 mm dish, or six-well plate, remove media by gentle aspiration and add 1 mL ice cold 0.5 M TCA. Incubate cells on ice for 5 minutes. Scrape the cells from flask with additional 0.5 M TCA if needed and transfer to a 2-5 mL centrifuge tube. Centrifuge at 3000 RPM (approximately 900-1,000 RCF) for 7 minutes at 4°C. Discard the supernatant. The remaining steps are performed at room temperature.
   b. For non-adherent cells in a 25 cm2 flask, 35 mm dish, or six-well plate, collect cells into 2-5 mL centrifuge tube, spin the cells down, decant media, add ice cold 0.5 M TCA and vortex. Incubate cells on ice for 5 minutes. Centrifuge at 3000 RPM (approximately 900-1,000 RCF) for 7 minutes at 4°C. Discard the supernatant. The remaining steps are performed at room temperature.

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2. Wash Pellet
   Add 1 mL 5% TCA/ 1 mM EDTA to the pellet. Vortex for 30 seconds. Centrifuge at 3000 RPM for 5 minutes. Discard the supernatant. Repeat wash one more time.

3. Extract neutral lipids
   Add 1 mL MeOH : CHCl3 (2:1) and vortex for 10 minutes at room temperature. Centrifuge at 3000 RPM for 5 minutes, discard the supernatant. Repeat neutral lipids extraction one more time. A small white pellet should be visible after this step.

4. Extract acidic lipids
   Add 750 µL MEOH:CHCl3:1HCl (80:40:1) and vortex for 25 minutes at room temperature. Centrifuge at 3000 RPM for 5 minutes. Transfer supernatant to a new 2 mL vial. Discard pellet.

5. Phase split
   To supernatant from step 4, add 250 µL of CHCl3 and 450 µL of 0.1 N HCl. Vortex for 30 seconds. Centrifuge at 3000 RPM for 5 minutes to separate organic and aqueous phases. Disregard any excess cellular debris that may appear between the two layers. Collect 0.5 mL of the organic (lower) phase, with a positive displacement pipette, into a new 1.5 mL vial and dry in a vacuum dryer (45-60 minutes). Dried lipid can be stored at -20°C for up to 12 months. The dried lipid should not be visible. If there is a visible substance at the end of this step, it is most likely cell debris that was not eliminated in the extraction. Do not attempt to dissolve the cell debris in the detection assay buffer (step 1c) this black to yellow substance should also be

References

PI(4)P Background References

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Lipid Extraction Protocol References

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K-4000E References

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Related Products

Products	Catalog Number
Other PIP Mass Assays	
PIP_3 Mass ELISA	K-2500s
PI(3)P Mass ELISA	K-3300
$PI(3,4)P_2$ Mass ELISA	K-3800
$PI(4,5)P_2$ Mass ELISA	K-4500
Activity Assays	
PI3-Kinase Activity ELISA	K-1000s
PTEN Activity ELISA	K-4700
PI(4)P 5-Kinase Activity Assay	K-5700
PI(4)P Binding Proteins and Antibodies	
PI(4)P Grip	G-0402
Purified Anti-PtdIns(4)P IgM	Z-P004

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