PI(3)P Mass ELISA

K-3300 (96 tests)

Support: echelon@echelon-inc.com

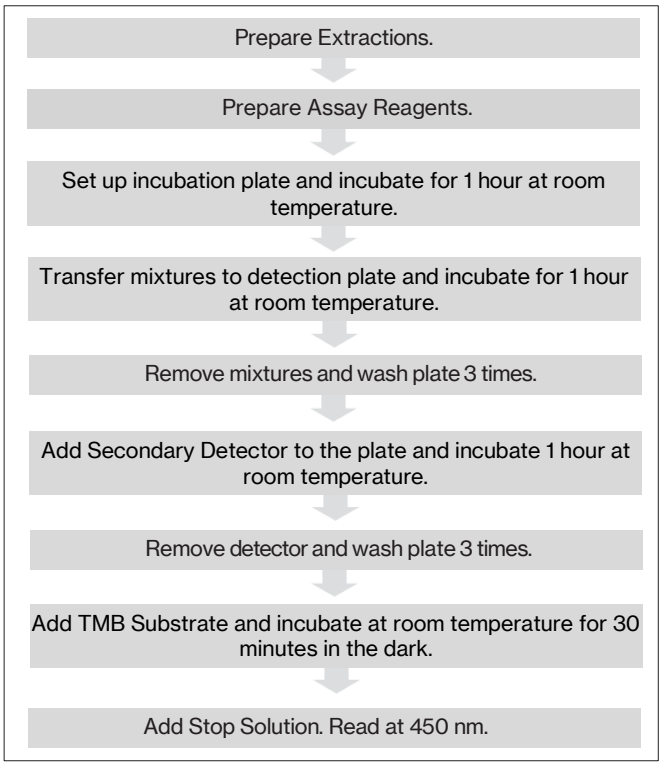
Description: The PI(3)P Mass ELISA measures the amount of PI(3)P extracted from cells by means of a competitive ELISA.

Storage: The kit comes in two parts with different storage requirements. Upon receipt store Kit Part 1 at 4ºC and Kit Part 2 at -20ºC. Store prepared reagents as indicated in the protocol.

Materials Provided

Catalog #	Description	Quantity
K-3001	PI(3)P Detection Plate (Strip-well)	1 plate
K-3302	PI(3)P Standard	12.2 µg
K-3305	PI(3)P Detector	2 vials
K-SEC2	Secondary Detector	300 µL
K-PBST2	10x PBS-T	20 mL
K-GS01	Protein Stabilizer	2 x 600 µL
K-TMB1	TMB Solution	12 mL
K-STOPt	1 N H2SO4 Stop Solution	8 mL
---	Yellow 96-well polypropylene plate	1 plate
---	Plate Sealers	2 seals

Quick Protocol



Additional Materials Provided by User

- Extracted PI(3)P samples (See PI(3)P Extraction Protocol at the end of this document)
- Buffers and solvents for PI(3)P extractions: Trichloroacetic Acid, EDTA, Methanol, Chloroform, and 12 N HCl
- Vacuum dryer
- 450 nm absorbance plate reader

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Background

Phosphatidylinositol 3-phosphate, or PI(3)P, is a product of Class III PI3-Kinase (Vps34) found in endocytic membranes and is the substrate for dephosphorylation by myotubularin. PI(3)P helps to recruit signal proteins to various cellular membranes that are involved in protein trafficking.

Assay Design

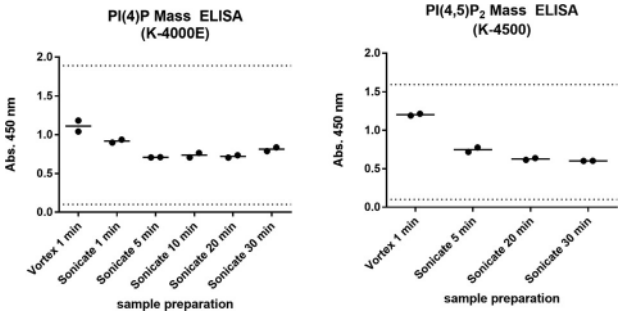
The assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(3)P present. Once PI(3)P has been extracted from cellular samples, it is incubated with the PI(3)P Detector, then added to the PI(3)P Detection Plate for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric substrate is used to detect PI(3)P detector protein bound to the plate. The colorimetric signal is read at 450 nm absorbance. The optical density is inversely proportional to the amount of PI(3)P extracted from cells. The assay is sensitive to about 1 pmol PI(3)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 without shaking. A reduction in signal and some loss in sensitivity may be observed.
2. The PI(3)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 "FAQ - PIP Mass Assays" 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 (examples are shown in the graphs below); 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 different. It is suggested that your sonicator is tested with PI(3)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.



6. For PI(3)P extraction from tissue samples, measure the weight of the tissue sample first as cell number normalization basis, flash freeze the tissue sample in liquid nitrogen, ground to powder on dry ice, then proceed extraction as a cell pellet.
7. If the amount of PI(3)P observed in your sample is outside of our assay range, we recommend decreasing the number of cells before increasing the quadruplicate dilution factor of the cell extraction samples.

Protocol for the detection of PI(3)P

Please read this entire section and the assay notes section before beginning the assay. Prior to use, place PI(3)P Detector (K-3305) and Secondary Detector (K-SEC2) on ice and bring all other kit components and the extracted PI(3)P samples to room temperature.

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. PBS-T 3% Protein Stabilizer (PBS-T 3%PS): For the entire plate, prepare PBS-T 3%PS by adding 540 µL Protein Stabilizer (K-GS01) to 18 mL PBS-T. Vortex briefly. Leave PBS-T 3%PS at room temperature until use. Prepare only the volume needed for the current assay. Reserve the remainder of undiluted Protein Stabilizer for dilution of the secondary detector.

PI(3)P Mass ELISA General Protocol

1. PI(3)P Standard
   a. Prepare a 400 pmol PI(3)P Standard stock by adding 1.6 mL of PBS-T 3%PS to the vial of PI(3)P Standard (K-3302). Vortex for at least 1 minute to rehydrate the lipid. Spin down and leave vial at room temperature before use.
   b. Make five, 4-fold serial dilutions from the 400 pmol standard stock by adding 100 µL of the 400 pmol PI(3)P standard stock or previous dilution to 300 µL of PBS-T 3%PS. The remaining portion of the 400 pmol PI(3)P standard stock can be stored at -20ºC for up to 3 months.

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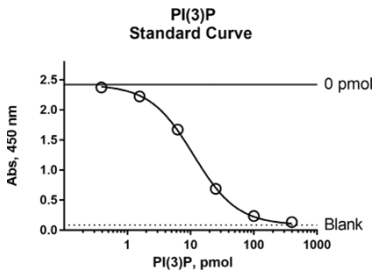
2. Rehydrate PI(3)P extraction samples with PBS-T 3%PS. We suggest adding 125 µL to 245 µL for duplicate, triplicate, or quadruplicate wells (See PI(3)P Extraction Protocol). Sonicate 5-10 min in a room temperature water bath – no ice. Vortex, spin down samples and leave at room temperature. Stimulated cells may need higher dilutions. Dilute extraction samples as necessary. For additional guidance on sample preparation see the assay notes section.
3. Reconstitute PI(3)P Detector (K-3305) by adding 1 mL PBS-T 3% PS. Incubate on ice for a few minutes. Use same day. Proceed to the next step while it rehydrates.
4. Prepare the Yellow 96-well Incubation Plate. We recommend running extractions, controls, and standards in duplicate or triplicate. A sample layout is provided.
   a. Add 60 µL/well of each standard (400 to 0.39 pmol) in duplicate or triplicate to rows B–G.
   b. Add 60 µL/well PBS-T 3% PS to 0 pmol control wells in row H.
   c. Add 120 µL/well PBS-T 3% PS to blank control wells in row A (no PI(3)P Detector or lipid added).
   d. Add 60 µL/well of cell extraction samples in duplicate or triplicate.
   e. Gently mix the rehydrated PI(3)P Detector (step 3) by inverting or flicking the vial, then briefly spin down. Pipet up and down to mix, then dilute 0.8 mL of Detector into 7.2 mL PBS-T 3% PS. Mix the dilution gently by inverting. Add 60 µL/well of diluted Detector to all sample, standard, and 0 pmol control wells – excluding blank wells in row A.
   f. Seal the plate and incubate on a shaker at room temperature for 1 hour.
5. After incubation, transfer 100 µL/well from the incubation plate to the corresponding well of the Detection Plate (K-3001) using a multi-channel pipettor. Seal and shake at room temperature for 1 hour.
6. Wash the Detection Plate 3 times with 200 µL/well PBS-T.
7. Briefly spin down Secondary Detector (K-SEC2). Dilute 1:100 in fresh PBS-T 3%PS (120 µL K-SEC2 + 360 µL K-GS01 to 12 mL PBS-T for one plate). Prepare only what's needed for the current assay. Store leftover Detector and Stabilizer at 4 °C.
8. Add 100 µL of diluted Secondary Detector to each well of the Detection Plate. Seal the plate and incubate on a plate shaker at room temperature for 1 hour.
9. Wash the Detection Plate 3 times with 200 µL/well PBS-T.
10. Add 100 µL of TMB solution (K-TMB1) to each well. Allow color to develop for 30 minutes in dark (or cover plate with aluminum foil).
11. Stop color development by adding 50 µL of 1 N H2SO4 Stop Solution (K-STOPt) to each well. Blue will change to yellow upon addition of stop solution. Eliminate any big air bubbles present in wells before reading the plate.
12. Read absorbance at 450 nm on a plate reader.

Data Analysis

Cellular PI(3)P levels are estimated by comparing sample values to a standard curve. Plot absorbance vs. pmol PI(3)P to generate the standard curve. Sample values are interpolated from this curve. The standard curve shown here was fitted using non-linear regression in GraphPad Prism, applying a sigmoidal dose-response (4-parameter logistic, 4PL) model with curve top and bottom constrained to 0 pmol and Blank, respectively.



	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Sample 1	Sample 1	Sample 1	Sample 9	Sample 9	Sample 9	Sample 17	Sample 17	Sample 17
B	400 pmol Standard	400 pmol Standard	400 pmol Standard	Sample 2	Sample 2	Sample 2	Sample 10	Sample 10	Sample 10	Sample 18	Sample 18	Sample 18
C	100 pmol Standard	100 pmol Standard	100 pmol Standard	Sample 3	Sample 3	Sample 3	Sample 11	Sample 11	Sample 11	Sample 19	Sample 19	Sample 19
D	25 pmol Standard	25 pmol Standard	25 pmol Standard	Sample 4	Sample 4	Sample 4	Sample 12	Sample 12	Sample 12	Sample 20	Sample 20	Sample 20
E	6.25 pmol Standard	6.25 pmol Standard	6.25 pmol Standard	Sample 5	Sample 5	Sample 5	Sample 13	Sample 13	Sample 13	Sample 21	Sample 21	Sample 21
F	1.56 pmol Standard	1.56 pmol Standard	1.56 pmol Standard	Sample 6	Sample 6	Sample 6	Sample 14	Sample 14	Sample 14	Sample 22	Sample 22	Sample 22
G	0.39 pmol Standard	0.39 pmol Standard	0.39 pmol Standard	Sample 7	Sample 7	Sample 7	Sample 15	Sample 15	Sample 15	Sample 23	Sample 23	Sample 23
H	0 pmol Standard	0 pmol Standard	0 pmol Standard	Sample 8	Sample 8	Sample 8	Sample 16	Sample 16	Sample 16	Sample 24	Sample 24	Sample 24

Support Protocol: Lipid Extraction of PI(3)P from cells

The PI(3)P extraction protocol was verified with 5 x 10^6 NIH-3T3 cells (80% confluence) per T-75 flask. The number of cells necessary for PI(3)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 "FAQ - PIP Mass Assays" 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

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(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(3)P from cells

1. Collect Cells
   a. For adherent cells in a 75 cm2 flask, remove medium by gentle aspiration and immediately add 5 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 15 mL centrifuge tube. Centrifuge at 3000 RPM (approximately 900-1,000 RCF) for 7 minutes at 4ºC. Discard the supernatant. The remaining steps are performed at room temperature.
   b. For non-adherent cells in a 75 cm2 flask, collect cells into 15 mL centrifuge tube, spin the cells down, decant media, add 5 mL 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 4ºC. Discard the supernatant. The remaining steps are performed at room temperature.
2. Wash Pellet: add 3 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 3 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 may be visible after this step.
4. Extract acidic lipids: Add 2.25 mL MeOH : CHCl3 : 12 N HCl (80:40:1) and vortex for 25 minutes at room temperature. Centrifuge at 3000 RPM for 5 minutes. Transfer the supernatant to a new 15 mL centrifuge tube. Discard the pellet.
5. Phase split: To supernatant from step 4, add 0.75 mL of CHCl3 (avoid using plastic pipette tip) and 1.35 mL 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 organic (lower) phase, preferably with a positive displacement pipette, into a clean 1.5 - 2 mL vial and dry in a vacuum dryer (45 – 60 min). Dried lipid can be stored at -20ºC for up to 1 year.

References

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