Echelon Biosciences Inc.

PI(3,4)P_2 Mass ELISA

K-3800 (96 tests)

Support: echelon@echelon-inc.com

Description: 96-well ELISA Assay for Detection and Quantification of PI(3,4)P2 from

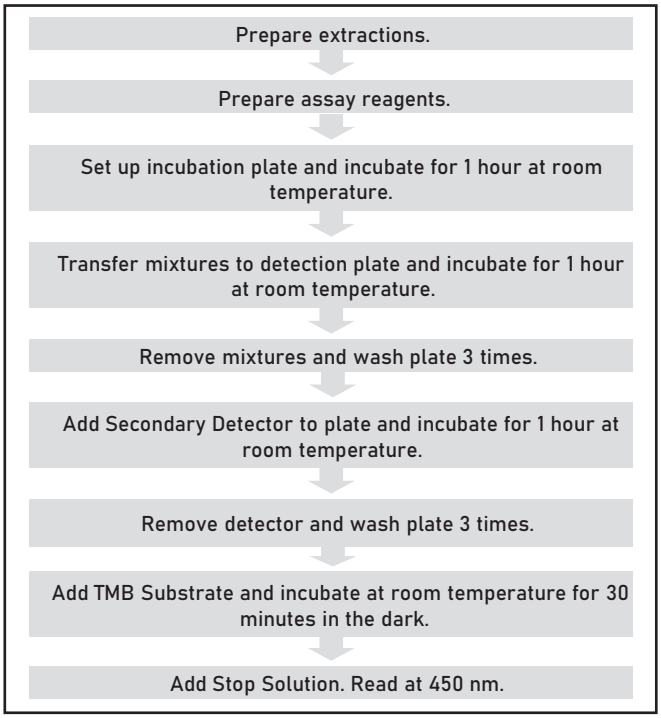
Materials Provided

Catalog #	Description	Amount
K-3801	PI(3,4)P2 Coated Strip-well Detection Plate	1 plate
K-3802	PI(3,4)P2 Standard	10.4 µg
K-3402	PI(3,4)P2 Detector	20 µL
K-SEC1	Secondary Detector	300 µL
K-PBST2	10 x PBS-T	20 mL
K-GS01	Protein Stabilizer	2x600 µL
K-TMB1	TMB Solution	12 mL
K-STOPt	1 N H_2SO_4 Stop Solution	10 mL
---	Colored 96-well polypropylene U-bottom plate	1 plate
---	Plate sealers	3 seals

Additional Materials Provided by User

- Extracted PI(3,4)P2 samples (See Support Protocol for PI(3,4)P2 extraction at the end of this document)
- Buffers and solvents for PI(3,4)P2 extractions: Trichloroacetic Acid, EDTA, Methanol, Chloroform, and 12 N HCl
- Vacuum dryer
- 450 nm absorbance plate reader

Quick Protocol



Storage Upon receipt, store kit at 4°C. Under proper storage conditions, the kit components are stable for 6 months. Centrifuge vials before opening.

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Background

Phosphatidylinositol 3,4-bisphosphate, or PI(3,4)P2, is an important second messenger produced by Class II PI3-Kinases and/or lipid phosphatase SHIP. Binding of PI(3,4)P2 by AKT/PKB is reported to assist in activation of this important serine/threonine kinase.

Assay Design

The assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(3,4)P2 produced. Once PI(3,4)P2 has been extracted from cell samples, it is incubated with a PI(3,4)P2 detector protein, then added to the PI(3,4)P2-coated plate for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric substrate is used to detect PI(3,4)P2 detector protein bound to the plate. The colorimetric signal is read at absorbance 450 nm and is inversely proportional to the amount of PI(3,4)P2 extracted from cells. The assay is sensitive to about 1

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. PI(3,4)P2 extraction samples can be sonicated 5-10 min in a room temperature water bath. 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 different. It is suggested that your sonicator is tested with PI(3,4)P2 extraction samples for day to day variation and time dependent consistency. If running other lipid mass assays you may want to consider dissolving your samples by the same method. How you dissolve the lipid will affect how well it goes into solution and can cause inconstancies in your data if it is not held constant.
2. Never let the detection plate dry out after the assay has started. Always have the next solution ready before discarding the current one from wells in use.
3. Use caution when using acid stop solution.
4. If the amount of PI(3,4)P2 observed in your sample is outside of our assay range, we recommend decreasing the number of cells before increasing to a quadruplicate dilution factor of the cell extraction samples.

Protocol for the detection of PI(3,4)P2

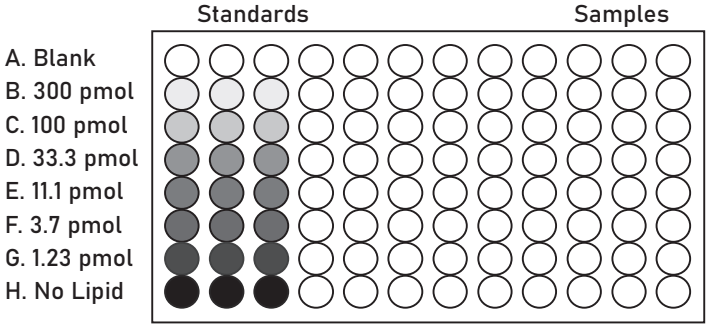
Please read this entire section and the assay notes section before beginning the assay. Prior to use, place PI(3,4)P2 Detector (K-3402), Secondary Detector (K-SEC1), and Protein Stabilizer (K-GS01) on ice and leave all other kit components and extracted PI(3,4)P2 samples at room temperature.

Reagent Preparation

1. PBS-T Buffer
   Prepare 1x PBS-T Buffer by diluting 20 mL of the 10x PBS-T Buffer (K-PBST2) with 180 mL dH2O. Keep prepared buffer at room temperature.
2. PBS-T 3% Protein Stabilizer (PS)
   Prepare PBS-T 3% PS. When using the entire plate, add 450 µL Protein Stabilizer (K-GS01) to 15 mL PBS-T. Vortex briefly. Leave PBS-T 3% PS buffer at room temperature. Make only the you will use for the current assay and store the remainder of the undiluted Protein Stabilizer at 4°C for future use.
3. PI(3,4)P2 Standards
   a. Prepare a 900 pmol PI(3,4)P2 Standard stock by adding 530 µL of PBS-T 3% PS to one vial of room temperature PI(3,4)P2 Standard (K-3802). Vortex for at least 60 seconds at maximum speed to reconstitute the lipid. Spin down and keep vial at room temperature before use.
   b. Make six, 3-fold serial dilutions from the 900 pmol stock by adding 120 µL of previous dilution to 240 µL of PBS-T 3% PS. The unused portion of PI(3,4)P2 Standard stock can be stored at -20°C for up to 3 months.
4. PI(3,4)P2 Extraction Samples
   Prepare PI(3,4)P2 dried down extraction samples by dissolving in PBS-T 3% PS. We suggest adding 125 µL to 185 µL for duplicate or triplicate wells. See support protocol for extraction. Vortex for at least 1 minute. Spin down samples and leave them at room temperature before adding to the ELISA incubation plate. Stimulated cells may need higher dilutions. Dilute extraction samples as necessary.
5. PI(3,4)P2 Detector
   Briefly centrifuge the vial of PI(3,4)P2 Detector (K-3402). Dilute PI(3,4)P2 Detector 1:300 in PBS-T 3% PS (Add 20 µL to 6 mL for the entire plate). Make only the amount you will use for the current assay and store the remainder of the PI(3,4)P2 Detector at 4°C. PI(3,4)P2 Detector can be stored at -20°C for longer term. Keep diluted PI(3,4)P2 Detector on ice. Diluted PI(3,4)P2 Detector is only good for that day.

Plate Setup and Incubation

We suggest that extractions, controls, and standards be run in duplicate or triplicate. An example to set up the PI(3,4)P2 ELISA in the incubation plate is shown below.



6. Add to Incubation Plate
   a. Pipet 60 µL/well of each standard solution in duplicate or triplicate to rows B through G of incubation plate (colored plate).
   b. Pipet 60 µL/well of PBS-T 3% PS to the No Lipid control wells in row H of incubation plate.
   c. Pipet 120 µL/well of PBS-T 3% PS to the blank control wells in row A of incubation plate. (No PI(3,4)P2 detector or lipid will be added to these wells.)
   d. Pipet 60 µL/well cell extraction samples to incubation plate. Lipid extracts should be run in duplicate or triplicate.
   e. Pipet 60 µL/well of diluted PI(3,4)P2 detector to all sample, standard, and no lipid control wells except the blank controls in row A.
   f. Seal the incubation plate with a plate sealer and incubate

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7. Following incubation, transfer 100 µL from each well to the corresponding well in the Detection Plate (K-3801). This can easily be accomplished with a multi-channel pipettor. Seal the plate with a plate sealer and incubate on a plate shaker at room temperature for 1 hour.

8. Discard the solution from the Detection Plate. Wash the plate 3 times with 200 µL/well PBS-T.

9. Prepare Secondary Detector
   a. Briefly centrifuge the vial of Secondary Detector (K-SEC1). Dilute the Secondary Detector 1:100 with PBS-T 3% PS (Add 120 µL Secondary Detector and 360 µL protein stabilizer (K-GS01) to 12 mL PBS-T for the entire plate). Invert the tube multiple times to mix solution gently. Dilute only the amount you will use for the current assay and store the remainder of the Secondary Detector at 4°C for future
   b. use.
   Add 100 µL of diluted Secondary Detector to each well of
   c. the detection plate.
   Seal the plate and incubate on a plate shaker at room temperature for 1 hour.

10. Discard the solution from the Detection Plate. Wash the plate 3 times with 200 µL/well PBS-T.

11. Detection
   a. 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).
   b. Stop color development by adding 50 µL of 1 N H_2SO_4 stop solution (K-STOPt) to each well. Blue color will change to yellow color 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(3,4)P2 quantities can be estimated by comparing the values from the wells containing PI(3,4)P2 extraction samples to the values in the standard curve. Plot the absorbance values obtained vs. amount of PI(3,4)P2 per standard to generate a standard curve. Determine the values of the PI(3,4)P2 in extraction samples by interpolating unknowns from the PI(3,4)P2 standard curve. A typical PI(3,4)P2 standard curve is shown in Figure 1.

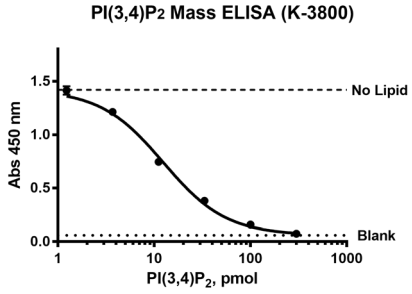

Figure 1.
PI(3,4)P2 standard curve was generated using non-linear regression analysis with GraphPad Software. A semi-log Sigmoidal dose-response with variable slope (four-parameter logistic, 4PL) analysis was utilized. For best results, constrain standard curve top and bottom using the No Lipid control signal and Blank control signal, respectively.

Support Protocol: Lipid extraction

Please read through entire protocol before beginning the extraction. PI(3,4)P2 extraction protocol as verified with 5 x 10^6 NIH-3T3 mouse fibroblast cells (75 cm^2 flask at 80% confluence). Larger or smaller amounts of cells require proportional adjustments of volumes. The amount of cells necessary for PI(3,4)P2 quantification needs to be determined for each cell type.

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(3,4)P2 from cells

1. Collect Cells
   a. For adherent cells in a 75 cm^2 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 the 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 4°C. Discard the supernatant. The remaining steps are performed at room temperature.
   b. For non-adherent cells in a 75 cm^2 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 4°C. 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

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Add 2.25 mL MeOH:CHCl3: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

References

1. Mondal et al., Phosphoinositide lipid phosphatase SHIP1 and PTEN coordinate to regulate cell migration and adhesion, Mol. Biol. Cell (2012) Vol. 23 No. 7, 1219-1230.
2. Costa et al., Measurement of PIP3 Levels Reveals an Unexpected Role for p110β in Early Adaptive Responses to p110α-Specific Inhibitors in Luminal Breast Cancer, Cancer Cell (2015), 27(1):97-108
3. A. Gray, H. Olsson, I. H. Batty, L. Priganica, and C. P. Downes, Nonradioactive methods for the assay of phosphoinosited 3-kinases and phosphoinositide phosphatases and selective detection of signaling lipids in cell and tissue extracts, Analyti-

Related Products

Products	Catalog Number
Other PIP Mass ELISAs	
PI(3)P Mass ELISA Kit	K-3300
PI(4)P Mass ELISA Kit	K-4000E
PI(4,5)P_2 Mass ELISA Kit	K-4500
PI(3,4,5)P_3 Mass ELISA Kit	K-2500s
Enzymes and Activity Assays	
SHIP2 Enzyme, PTEN Enzyme	E-1000, E-3000
PIP3 Phosphatase Activity Kit PTEN Activity ELISA	K-1400 K-4700
PIP2 Antibodies	
Anti-PtdIns(3,4)P_2 Antibody Biotinylated Anti-PtdIns(3,4)P2 Antibody	Z-P034, Z-P034b Z-B034

Please visit our website at www.echelon-inc.com for more phospholipid products.

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