

Echelon Biosciences Inc.

PI(3)P Mass ELISA Kit

K-3300 (96 tests)

Support: echelon@echelon-inc.com

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

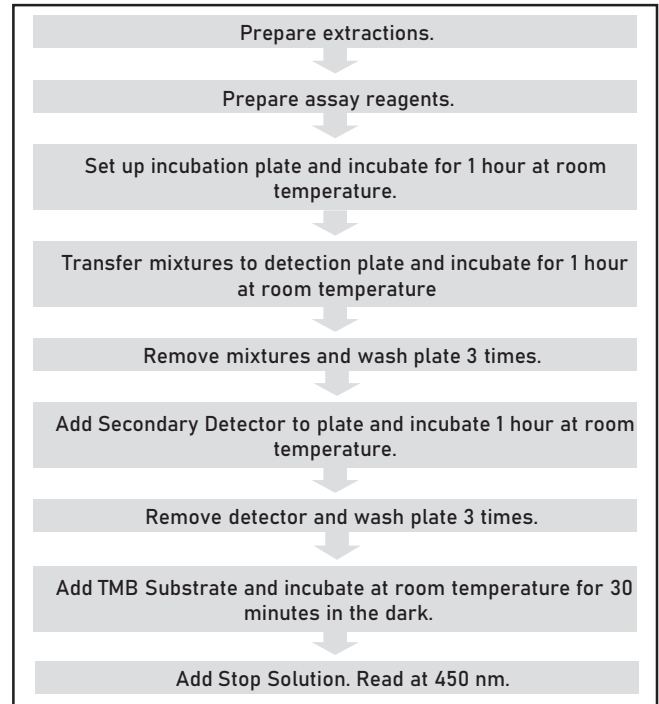
Materials Provided

Catalog #	Description	Amount
K-3001	PI(3)P Coated Strip-well Detection Plate	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	2x600 µL
K-TMB1	TMB Solution	12 mL
K-STOPt	1 NH ₂ SO ₄ Stop Solution	10 mL
---	Colored 96-well polypropylene U-bottom plate	1 plate
---	Plate sealers	2 seals

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

Quick Protocol



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 -20°C. Store prepared reagents as indicated in the protocol.

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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

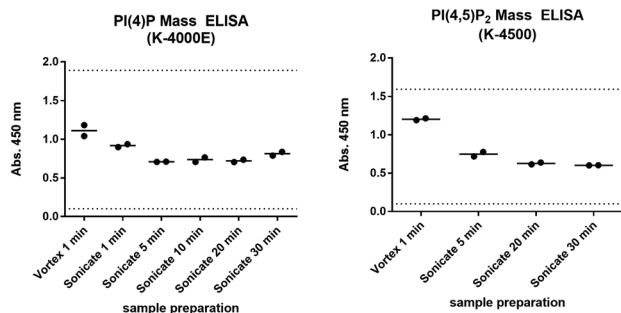
The assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(3)P produced. Once PI(3)P has been extracted from cellular samples, it is incubated with a PI(3)P detector protein, then added to the PI(3)P-coated plate for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric substrate is used to detect PI(3)P detector protein bound to the plate. The colorimetric signal is read at absorbance 450 nm and 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 inconsistencies 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 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 μ L Protein Stabilizer (K-GS01) to 18 mL PBS-T. Vortex briefly. Leave PBS-T 3%PS buffer at room temperature until use. Make ONLY the amount you will use for the current assay. The remainder of the undiluted Protein Stabilizer will be used again for the dilution of the secondary detector later.

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 μ L of the 400 pmol PI(3)P standard stock or previous dilution to 300 μ L of PBS-T 3%PS. The remaining portion of the 400 pmol PI(3)P standard stock can be stored at -20°C for up to 3 months.
2. PI(3)P Extracted Samples

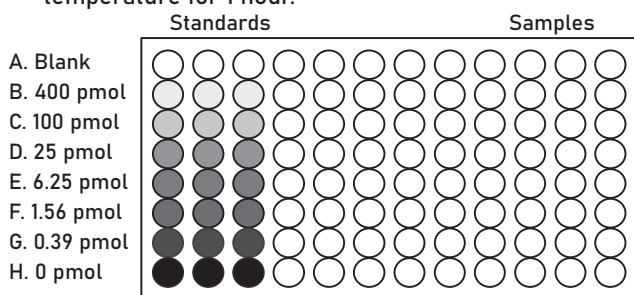
Prepare PI(3)P extraction samples by rehydrating in PBS-T 3%PS. We suggest adding 125 μ L to 245 μ L for duplicate, triplicate, or quadruplicate wells. (See PI(3)P Extraction Protocol) Sonicate samples for 5-10 min in a room temperature water bath. Do not add ice to water bath. See assay notes section for more information on sample preparation. Vortex, spin down samples and leave at room temperature before adding to the ELISA incubation plate. Stimulated cells may need higher dilutions. Dilute extraction samples as necessary.
3. PI(3)P Detector

Add 1 mL of PBS-T 3%PS to 1 vial of PI(3)P Detector (K-3305) to reconstitute protein. Leave the vial on ice for a few minutes to allow protein to rehydrate. Reconstituted PI(3)P Detector is only good for that day. While waiting, proceed to next step.
4. Set up colored Incubation Plate. We suggest that extractions, controls, and standards be run in duplicate or triplicate. An example to set up the PI(3)P ELISA in the incubation plate is shown on the following page.
 - a. Add 60 μ L/well of each standard solution (400 pmol through 0.39 pmol) in triplicate or duplicate to rows B

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- through G of the Incubation Plate (colored plate).
- Add 60 μL /well of PBS-T 3%PS to the 0 pmol control wells in row H of Incubation Plate.
 - Add 120 μL /well of PBS-T 3%PS to the blank control wells in row A of Incubation Plate. (No PI(3)P detector or lipid will be added to these wells.)
 - Add 60 μL /well cell extraction samples to Incubation Plate. Lipid extracts should be run in duplicate or triplicate.

Invert the vial of rehydrated PI(3)P Detector (step 3) a few times or flick the vial with finger to mix the detector gently, then spin down. Pipet up and down multiple times to mix and dilute 0.8 mL of this PI(3)P Detector into 7.2 mL of PBS-T 3%PS. Invert the tube a few times to mix gently. Add 60 μL /well of the diluted PI(3)P detector to 0 pmol control, all sample and standard wells. Do not add to blank controls in row A. Seal the incubation plate with a plate sealer and incubate on a plate shaker at room temperature for 1 hour.

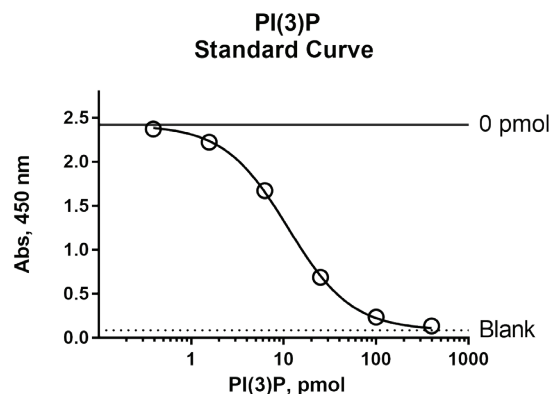


- Following the incubation, mix and transfer 100 μL from each well to the corresponding well in the Detection Plate (K-3001). This can easily be accomplished with a multi-channel pipettor. Seal the plate with a plate sealer and incubate on a plate shaker at room temperature for 1 hour.
- Wash the Detection Plate 3 times with 200 μL /well PBS-T.
- Secondary Detector**
 - Briefly centrifuge the vial of Secondary Detector (K-SEC2). Dilute the Secondary Detector 1:100 with fresh PBS-T 3%PS (Add 120 μL K-SEC2 and 360 μL K-GS01 to 12 mL PBS-T for the entire plate). Dilute ONLY the amount you will use for the current assay and store the remainder of the Secondary Detector and Protein Stabilizer at 4°C for future use.
 - 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.
- Wash the Detection Plate 3 times with 200 μL /well PBS-T.
- Detection**
 - 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).
 - Stop color development by adding 50 μL of 1 N H₂SO₄ 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.
- Read absorbance at 450 nm on a plate reader.

Data Analysis

Cellular PI(3)P quantities can be estimated by comparing the values from the wells containing PI(3)P extraction samples to the values in the standard curve. Plot the absorbance values obtained vs. amount of PI(3)P per standard to generate a standard curve. Determine the values of PI(3)P in extraction samples by interpolating unknowns

from the PI(3)P standard curve. The standard curve shown here was generated using non-linear regression analysis with GraphPad Prism software. A sigmoidal dose response-variable slope curve (four-parameter logistic, 4PL) analysis was utilized, with curve top and curve bottom constrained to 0 pmol and Blank, respectively.



Support Protocol: Lipid Extraction

Extraction of PI(3)P from cells

The PI(3)P extraction protocol was verified with 5×10^6 NIH-3T3 cells (80% confluence) per T-75 flask. The amount 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

- 0.5 M TCA** For 50 mL, dissolve 4.08 g TCA (Trichloroacetic Acid) in dH₂O and bring volume to 50 mL.
- 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.
- MeOH:CHCl₃ (2:1)** For 60 mL, add 40 mL MeOH to 20 mL CHCl₃
 - Measure CHCl₃ with a glass pipette. Pure CHCl₃ may dissolve plasticware.
 - 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.
- MeOH:CHCl₃:HCl (80:40:1)** For 60 mL, combine 40 mL MeOH, 20 mL CHCl₃, and 0.5 mL 12 N HCl
 - Measure CHCl₃ with a glass pipette. Pure CHCl₃ may dissolve plasticware.
 - 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.
 - Use 12 N concentrated 36% - 38% HCl. Do not use diluted acid.
- 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

- Collect Cells**
 - 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



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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 4°C. 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 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**
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 -20°C for up to 1 year.

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Related Products

Products	Catalog Number
Other PIP Mass ELISAs	
PI(4)P Mass ELISA Kit	K-4000E
PI(3,4)P ₂ Mass ELISA Kit	K-3800
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	
Class III PI3-Kinase Activity ELISA Kit	K-3000
INPP4 Activity ELISA Kit	K-5200
INPP4A Enzyme, active	E-8000
PI(3)P Binding Protein and Antibodies	
PI(3)P Grip™ (GST-p40PX)	G-0302
Anti-PtdIns(3)P Antibody	Z-P003
Anti-hVps34 Antibody for IP (Immunoprecipitation)	Z-R015
Anti-hVps34 Antibody for WB (Western Blot)	Z-R016

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