

PIP3 Mass ELISA Kit

K-2500s (96 tests)

Support: echelon@echelon-inc.com

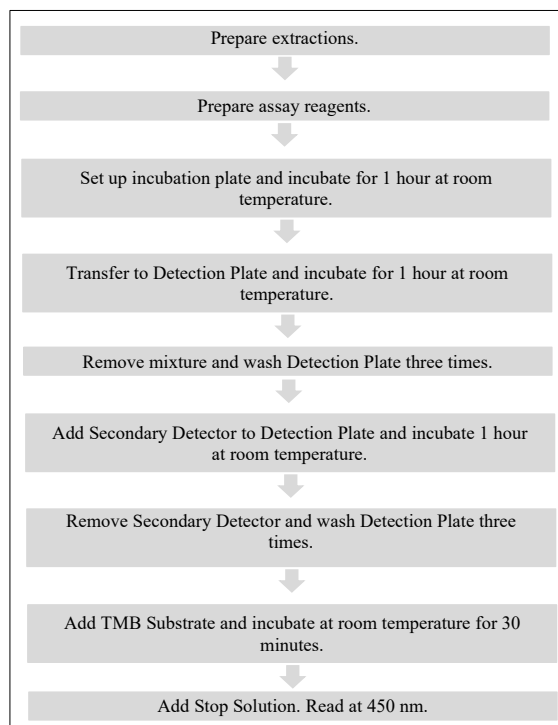
Description: The PIP3 Mass ELISA is a 96-well assay for detection and quantification of PI(3,4,5)P3 from cells.

Storage: Upon receipt store Kit Part 1 at 4°C and Kit Part 2 at -20°C.

Materials Provided

Catalog #	Description	Quantity
K-1001s	PI(3,4,5)P3 Coated Strip-well Detection Plate	1 plate
K-2501s	PI(3,4,5)P3 Standard	1.2 µg; 2 vials
K-2404	PI(3,4,5)P3 Detector	2 vials
K-SEC2	Secondary Detector	300 µL
K-PBST2	10x PBS-T Buffer	20 mL
K-GS01	Protein Stabilizer	600 µL
K-TMB1	TMB Solution	12 mL
K-STOPt	1 N H2SO4 Stop Solution	8 mL
Incubation Plate	Natural 96-well polypropylene Plate	1 plate
---	Clear acetate sheet, 1 side adhesive	2 pieces

Quick Protocol



Additional Materials Provided by User

- Extracted PIP3 samples (See Support Protocol: PIP3 Extraction at the end of this document)
- Buffers and solvents for PIP3 extractions: Trichloroacetic Acid, EDTA, Methanol, Chloroform, and 12 N HCl
- 450 nm absorbance plate reader
- Vacuum dryer

Hazardous Properties and Cautions: The toxicological and pharmacological properties of this compound are not fully known. For further information see the MSDS on request. This product is manufactured and shipped only in small quantities, intended for research and development in a laboratory utilizing prudent procedures for handling chemicals of unknown toxicity, under the supervision of persons technically qualified to evaluate potential risks and authorized to enforce appropriate health and safety measures. As with all research chemicals, precautions should be taken to avoid unnecessary exposure or risks.

Warranty and Disclaimer: Echelon warrants the product conforms to the specifications stated herein. In the event of nonconformity, Echelon will replace products or refund purchase price, at its sole option, and Echelon shall not be responsible for any other loss or damage, whether known or foreseeable to Echelon. No other warranties apply, express or implied, including but not limited to warranty of fitness for any purpose or implied warranty of merchantability. Purchaser is solely responsible for all consequences of its use of the product and Echelon assumes no responsibility therefore, including success of purchaser's research and development, or health or safety of any uses of the product.

Background

Phosphatidylinositol (3,4,5)-triphosphate (PI(3,4,5)P₃, PIP₃) is the product of Class I PI3-Kinase and is an important lipid second messenger with key roles in cellular proliferation and survival, most notable in activating the protein kinase AKT/PKB.

Assay Design

The assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(3,4,5)P₃ detected. Once PI(3,4,5)P₃ has been extracted from cells, it is incubated with a PI(3,4,5)P₃ detector protein, then added to the PI(3,4,5)P₃ coated plate for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric substrate is used to detect the PI(3,4,5)P₃ detector bound to the plate. The colorimetric signal is read at absorbance 450 nm and is inversely proportional to the amount of PI(3,4,5)P₃ extracted from cells. The assay is sensitive to 0.04 pmol PI(3,4,5)P₃.

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. PIP₃ extraction samples can be sonicated for 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 PIP₃ 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 inconsistencies in your data if it is not held constant.
2. For multiple uses of PIP₃ Detector (K-2404), reconstitute in 50 µL dH₂O on ice for a concentrated stock solution. Dilute the stock solution 1:300 into PBS-T 0.25% PS (see reagent preparation) prior to applying to

- plate. Store remaining PIP₃ Detector stock solution at -80°C and use within 1 month.
3. The PIP₃ 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. 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.
4. Use caution when using acidic Stop Solution.

PIP₃ Mass ELISA General Protocol

Please read this entire section and the assay notes before beginning. Prior to use, place PIP₃ Detector (K-2404) and Secondary Detector (K-SEC2) on ice. Leave all other kit components and extracted PIP₃ 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.
2. PBS-T 0.25% PS: Prepare PBS-T 0.25% PS. When using the entire plate, add 50 µL Protein Stabilizer (K-GS01) to 20 mL PBS-T. Vortex briefly. Leave PBS-T 0.25% PS at room temperature. Make only the amount you will use for the current assay and store the remainder of the undiluted Protein Stabilizer at 4°C for future use.
3. PIP₃ Standards
 - a. Prepare a 125 pmol PIP₃ standard solution by adding 400 µL of PBS-T 0.25% PS to one vial of PIP₃ Standard (K-2501s). Vortex for at least 60 seconds to suspend the lipid. Spin down and place vial at room temperature before use.
 - b. Make five, 5-fold serial dilutions from the 125 pmol PIP₃ standard by adding 60 µL of previous dilution to 240 µL PBS-T 0.25% PS.
 - c. The unused portion of PIP₃ standard stock can be stored at -20°C for up to 3 months.
4. PIP₃ Extraction Samples: Once PIP₃ is extracted and dried (see below for extraction protocol), suspend the samples in PBS-T 0.25% PS. We suggest adding 65 µL to 185 µL for single, duplicate, or triplicate wells. Vortex for at least 1 minute, briefly spin down the samples and place at room temperature. Stimulated cells may need

higher dilutions. Dilute extraction samples as necessary.

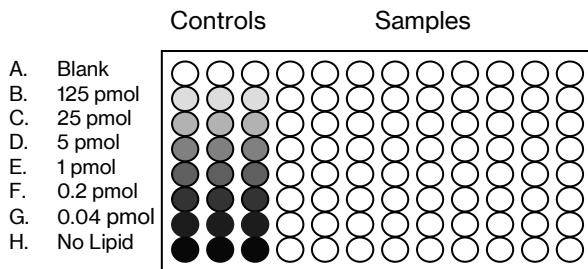
5. PIP3 Detector: Add 1 mL of PBS-T 0.25% PS to 1 vial of PIP3 Detector (K-2404) to reconstitute the detector protein. Mix gently by inverting vial multiple times. Centrifuge briefly and leave on ice for 5 to 10 min to allow detector to hydrate. This reconstituted PIP3 Detector is good for the day.

Protocol for the detection of PI(3,4,5)P3

We suggest that extractions be run in a single well or in duplicates while controls and standards be run in duplicates or triplicates. An example to set up the PIP3 ELISA in the Incubation Plate is shown below.

6. Incubation Plate Setup

- a. Pipet 60 μ L/well of each standard solution (Step 3) in duplicate or triplicate to rows B through G of the Incubation Plate



- b. Pipet 60 μ L/well of PBS-T 0.25% PS to the No Lipid control wells in row H of the Plate.
- c. Pipet 120 μ L/well of PBS-T 0.25% PS to the Blank control wells in row A of the Plate. (No PIP3 detector or lipid will be added to these wells.)
- d. Pipet 60 μ L/well cell extraction samples (Step 4) to Incubation Plate. Lipid extracts can be run in a single, duplicates, or in triplicates.
- e. Pipet up and down the reconstituted PIP3 detector (Step 5) multiple times. Further dilute 1:15 by adding 500 μ L of PIP3 Detector to 7 mL of PBS-T 0.25% PS. This is enough for the entire plate. Immediately pipet 60 μ L/well of diluted PIP3 Detector to all No Lipid control, standard, and sample wells. Do not add PIP3 detector to

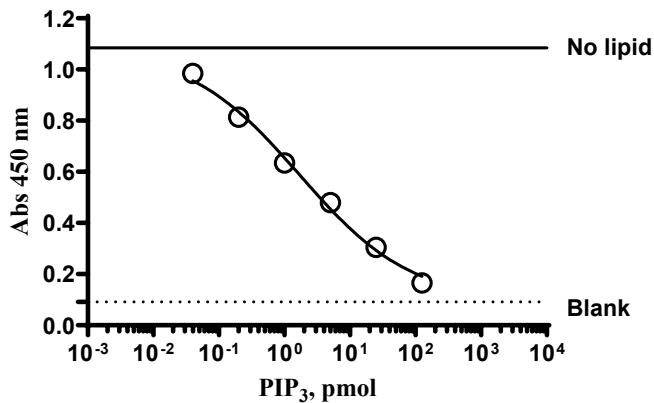
the Blank controls in row A.

- f. Seal the Incubation Plate with a plate sealer and incubate on a plate shaker at room temperature for 1 hour.
7. Following incubation, transfer 100 μ L from each well of the Incubation Plate to the corresponding well of the Detection Plate (K-1001s). This can 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. Following incubation, discard the solution from the Detection Plate. Wash the plate 3 times briefly with 200 μ L/well PBS-T.
9. Briefly spin down the vial of Secondary Detector (K-SEC2) and prepare by diluting Secondary Detector 1:150 with PBS-T Buffer. For example, for the entire plate add 80 μ L of Secondary Detector to 12 mL of PBS-T Buffer. Dilute only the amount you will use for the current assay and store the remainder of the undiluted Secondary Detector at 4°C for future use.
10. Discard the last wash from the Detection Plate. Add 100 μ L of diluted Secondary Detector to each well. Seal the plate and incubate on a plate shaker at room temperature for 1 hour.
11. Following incubation, discard the solution from the Detection Plate. Wash the plate 3 times briefly with 200 μ L/well PBS-T.
12. Discard the last wash from the Detection Plate completely. Add 100 μ L of TMB Solution (K-TMB1) to each well. Allow color to develop for 30 minutes in dark (or cover plate with aluminum foil).
13. Stop color development by adding 50 μ L of 1 N H₂SO₄ Stop Solution (K-STOPT) to each well. Blue color will change to yellow color upon addition of the 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 PIP3 quantities can be estimated by comparing the values from the wells containing PIP3 extraction samples to the values in the standard curve. Plot the absorbance values obtained vs. the amount of PIP3 per standard to generate a standard curve. Determine the values of PIP3 in PIP3 extraction samples by interpolating unknowns from the PIP3 standard curve. The standard curve shown below 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.

PIP₃ Standard Curve



PIP₃ standard curve was generated using non-linear regression analysis with GraphPad Software. A semi-log [Sigmoidal dose-response (variable slope)] 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 the entire protocol carefully before beginning the extraction. PIP₃ extraction protocol was verified with 20 x 10⁶ NIH-3T3 mouse fibroblast cells (T-150 cm² flask at 80% confluence). Larger or smaller amounts of cells require proportional adjustments of volumes. The number of cells necessary for PIP₃ quantification needs to be determined for each cell type.

Solutions for Extraction

- 0.5 M TCA:** For 100 mL, dissolve 8.16 g TCA (Trichloroacetic Acid) in dH₂O and bring volume to 100 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 PIP₃ from cells

- Collect Cells**
For adherent cells in a 150 cm² flask, remove media by gentle aspiration and immediately add 10 mL ice cold 0.5 M TCA. For non-adherent cells, spin the cells down, decant media, then add 10 mL ice cold 0.5 M TCA. Incubate cells on ice for 5 minutes. Scrape adherent cells from flask with additional 0.5 M TCA if needed and transfer to a 15 mL centrifuge tube. Centrifuge at 3000 RPM (approximately 900-1000 RCF) for 7 minutes at 4°C. Discard the supernatant. The remaining steps are performed at room temperature.
- 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.
- 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 lipid extraction one more time. A small white pellet should be visible after this step.
- Extract acidic lipids**
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 and transfer the supernatant to a new 15 mL centrifuge tube. The pellet is no longer needed.
- Phase Split**
To supernatant from step 4, add 0.75 mL of CHCl₃ (Avoid using plasticware to measure pure CHCl₃) 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 1.5 mL of the organic (lower) phase, preferably with a positive displacement pipette, into a new 1.5 - 2 mL vial and dry in a vacuum dryer (SpeedVac) at room temperature. Dried lipid can be stored at -20°C for up to 1 year.

References

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- A. Gray, H. Olsson, I. H. Batty, L. Priganica, and C. P. Downes, Nonradioactive methods for the assay of phosphoinositide 3-kinases and phosphoinositide phosphatases and selective detection of signaling lipids in cell and tissue extracts, *Analytical Biochemistry* 313(2003) 234-245.

Related Products

Products	Catalog Number
Other PIP Mass ELISAs	
PI(4,5)P ₂ Mass ELISA Kit	K-4500
PI(4)P Mass ELISA Kit	K-4000E
PI(3)P Mass ELISA Kit	K-3300
PI(3,4)P ₂ Mass ELISA Kit	K-3800
Enzymes and Activity Assays	
PI3-Kinase Activity Kits	K-1000s, K-3000
SHIP2 Enzyme	E-1000
PTEN Enzyme	E-3000
5'PIP3 Phosphatase Activity FP Kit	K-1400
PTEN Activity Kit	K-4700
PIP3 Binding Protein and Antibodies	
PI(3,4,5)P ₃ Grip	G-3901
Anti-PtdIns(3,4,5)P ₃ Antibodies	Z-P345, Z-P345b Z-G345

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