Echelon Biosciences Inc.

PIP_3 Mass ELISA Kit

K-2500s (96 tests)

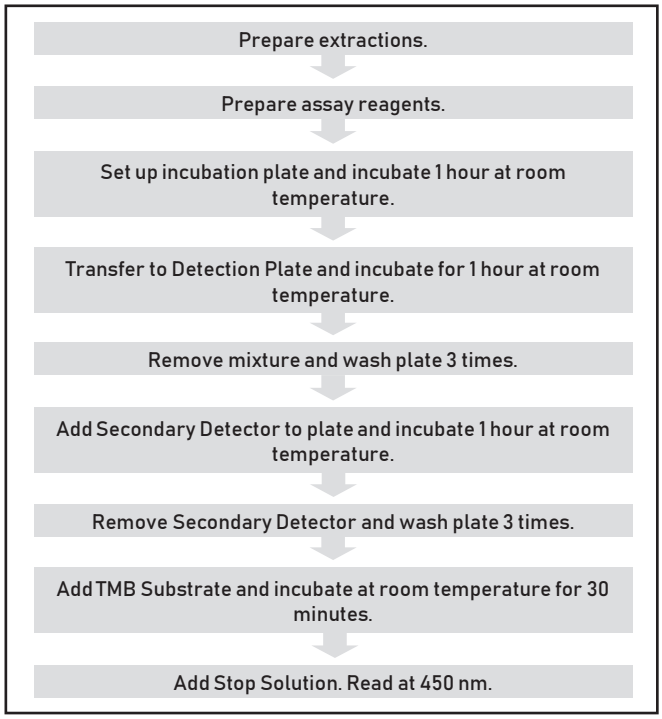
Support: echelon@echelon-inc.com

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

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 H_2SO_4 Stop Solution	10 mL
---	Colored 96-well polypropylene U-bottom 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

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 (3,4,5)-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 protein bound to the plate. The colorimetric signal is read at absorbance 450 nm and is inversely proportional to the amount of PI(3,4,5)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 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 inconstancies 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 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 of 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.

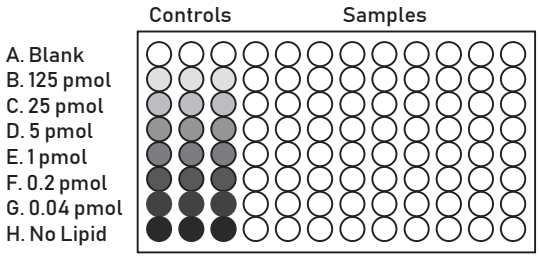
Prior to use, place PIP3 Detector (K-2404) and Secondary Detector (K-SEC2) on ice. Leave all other kit components and extracted PIP3 samples at room temperature.

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 re-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
   Prepare PIP3 extraction samples by re-suspending in PBS-T 0.25% PS. We suggest adding 65 µL to 185 µL for single, duplicate, or triplicate wells. See support protocol for phospholipids extraction. Vortex for at least 1 minute. Leave samples at room temperature. Spin down samples before adding to the Incubation Plate. 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 rehydrating. Reconstituted PIP3 Detector is only good for that 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 µL/well of each standard solution (Step 3) in duplicate or triplicate to rows B through G of the Incubation Plate (colored plate).
   b. Pipet 60 µL/well of PBS-T 0.25% PS to the No Lipid control wells in row H of the Plate.
   c. Pipet 120 µL/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 µL/well cell extraction samples (Step 4) to Incubation Plate. Lipid extracts can be run in a single well, duplicates, or in triplicates.
   e. Pipet up and down the reconstituted PIP3 detector (Step 5) multiple times. Further dilute 1:15 by adding 500 µL of PIP3 Detector to 7 mL of PBS-T 0.25% PS. This is enough for the entire plate. Immediately pipet 60 µL/well of diluted PIP3

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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 µL from each well of the Incubation Plate to the corresponding well of the Detection Plate (K-1001s). 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 briefly with 200 µL/well PBS-T.

9. Prepare Secondary Detector
   a. Briefly spin down the vial of Secondary Detector (K-SEC2). Dilute the Secondary Detector 1:150 with PBS-T Buffer (Add 80 µL of Secondary Detector to 12 mL of PBS-T Buffer for the entire plate). 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.
   b. Discard the last wash from the Detection Plate. Add 100 µL of diluted Secondary Detector to each well. 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 briefly with 200 µL/well PBS-T.

11. Detection
   a. Discard the last wash from the Detection Plate completely. 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 the 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 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.

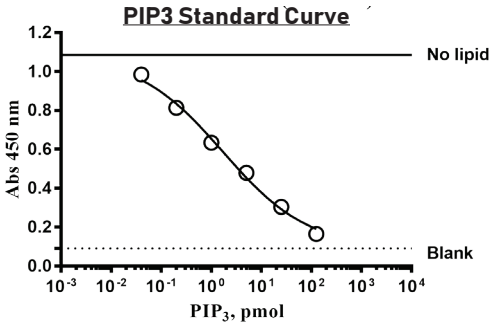

Figure 1. PIP3 standard curve was generated using non-linear regression analysis with GraphPad Software. A semi-log [Sigmoidal dose-response (variable slope)] analysis was utilized. For best results, constrain standard curve top and bottom using the No Lipid control signal and Blank control signal, respectively. TMB development time: 30 min

Support Protocol: Lipid Extraction

Please read through entire protocol carefully before beginning the extraction.

PIP3 extraction protocol as verified with 20 x 10^6 NIH-3T3 mouse fibroblast cells (T-150 cm2 flask at 80% confluence). Larger or smaller amounts of cells require proportional adjustments of volumes. The amount of cells necessary for PIP3 quantification needs to be determined for each cell type.

Solutions for Extraction

1. 0.5 M TCA
   For 100 mL, dissolve 8.16 g TCA (Trichloroacetic Acid) in dH2O and bring volume to 100 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 $CHCl_3$
   a. Measure $CHCl_3$ with a glass pipette. Pure $CHCl_3$ may dissolve plasticware.
   b. MeOH:$CHCl_3$ (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:$CHCl_3$:HCl (80:40:1)
   For 60 mL, combine 40 mL MeOH, 20 mL $CHCl_3$, and 0.5 mL 12 N HCl
   a. Measure $CHCl_3$ with a glass pipette. Pure $CHCl_3$ may dissolve plasticware.
   b. MeOH:$CHCl_3$: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 PIP3 from cells

1. 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.
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:$CHCl_3$ (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.
4. Extract acidic lipids
   Add 2.25 mL MeOH:$CHCl_3$: HCl (80:40:1) and vortex for 25 minutes at room temperature. Centrifuge at 3000 RPM for 5 minutes and

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transfer the supernatant to a new 15 mL centrifuge tube. The pellet is no longer needed.

5. 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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6. Kellie A. Mouchemore, et al., Specific inhibition of PI3K p110δ inhibits CSF-1-induced macrophage spreading and invasive capacity, FEBS J. (2013) 280(21): 5228–5236.
7. 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-Kinases, WT or Mutant	E-2000, P28-10H, P27-15H
PI3-Kinase Activity Kits	K-1000s, K-1100, K-1300, K-3000
SHIP2 Enzyme PTEN Enzyme	E-1000 E-3000
5'PIP3 Phosphatase Activity FP Kit PTEN Activity Kit	K-1400 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-A345 Z-P345, Z-P345b Z-B345, Z-B345b Z-G345, Z-H345

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