Echelon Biosciences Inc.

PI(4,5)P_2 Mass ELISA

K-4500 (96 tests)

Support: echelon@echelon-inc.com

Description: 96-well ELISA Assay for Detection and Quantification of PI(4,5)P2 from cells

Materials Provided

Catalog #	Description	Amount
K-4501	PI(4,5)P2 Coated Strip-well Detection Plate	1 plate
K-4502	PI(4,5)P2 Standard, diC16	1 vial
K-2302	PI(4,5)P2 Detector	2 vials
K-SEC2	Secondary Detector	1 vial
K-PTAB	PBS Tablet	1 tablet
K-PBST2	10x PBS-T	20 mL
K-GS01	Protein Stabilizer	1 vial
K-TMB1	TMB Solution	1 bottle
K-STOPt	1 N H2SO4 Stop Solution	1 bottle
---	Colored 96-well polypropylene U-bottom plate	1 plate
---	Plate sealers	2 seals

Quick Protocol

Prepare extractions.

Prepare assay reagents.

Set up incubation plate and incubate for 1 hour at room temperature.

Transfer mixtures to detection plate and incubate for 1 hour at room temperature.

Remove mixtures and wash plate 3 times.

Add Secondary Detector to plate and incubate for 1 hour at room temperature.

Remove detector and wash plate 3 times.

Add TMB Substrate and incubate at room temperature for 30 minutes in the dark.

Add Stop Solution. Read at 450 nm.

Additional Materials Provided by User

- Extracted PI(4,5)P2 samples (See Support Protocol: PI(4,5)P2 Extraction at the end of this document)
- Buffers and solvents for PI(4,5)P2 extractions: Trichloroacetic Acid, EDTA, Methanol, Chloroform, and 12 N HCl
- Vacuum Dryer
- 450 nm absorbance plate reader

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 4,5-bisphosphate (PI(4,5)P2, PIP2) is a substrate for Class I PI3-Kinase and the product of PTEN phosphatase reactions. It is a ubiquitous lipid shown to play a central role in a variety of cell functions including: recruitment of PH-domain containing proteins to membranes, binding non PH domain containing proteins (e.g., gelsolin and profilin), regulating actin polymerization, and for Phospholipase C-coupled G-protein pathways involved in intracellular calcium release.

Assay Design

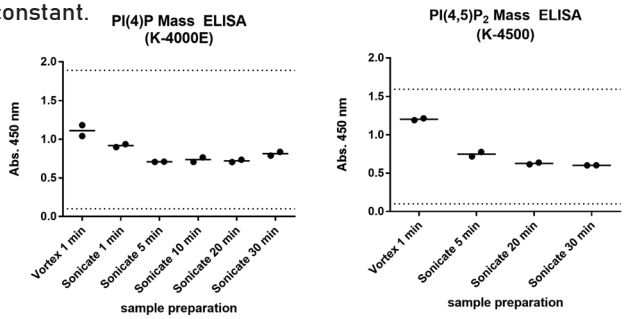
The assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(4,5)P2 detected. Once PI(4,5)P2 has been extracted from cellular samples, it is incubated with a PI(4,5)P2 detector protein, then added to the PI(4,5)P2-coated plate for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric substrate is used to detect PI(4,5)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(4,5)P2 extracted from cells. The assay is sensitive to about 0.02 pmol PI(4,5)P2.

Disclaimer

The PIP Mass Assays are used to quantify the total amount of a 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. Never let the detection plate dry out after the assay has started. Always have the next solution ready before discarding the current one including wash buffer from wells.
2. Use caution when using acid stop solution.
3. If the amount of PI(4,5)P2 observed in your sample is too high and outside of our assay range, we recommend decreasing the number of cells before increasing a quadruplicate dilution factor of the cell extraction samples.
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 (see graphs below). Variation in the number of vials, temperature of the water bath, and sonicator tuning can cause differences in sample performance. 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. Test your sonicator with PI(4,5)P2 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 in 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.

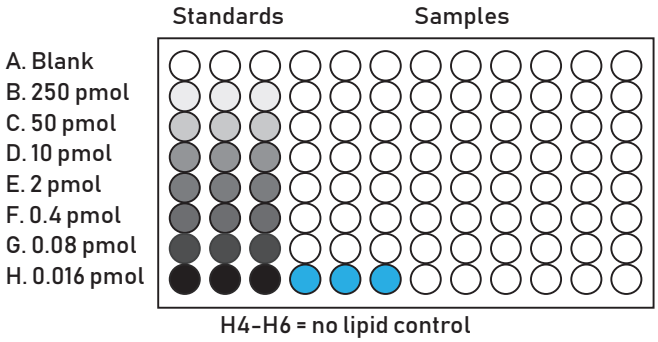


Protocol for the Detection of PI(4,5)P2

Please read the Assay Notes section, Assay Procedure, and Lipid Extraction Protocol, before beginning assay.

1. Place PI(4,5)P2 Detector (K-2302) and Secondary Detector (K-SEC2) on ice until use. Place all remaining kit reagents and PI(4,5)P2 extraction samples at room temperature until use. All remaining steps are performed at room temperature unless indicated otherwise.
2. PBS Prepare by dissolving the PBS tablet (K-PTAB) in 200 mL dH2O. PBS is stable at room temperature.
3. PBS-T Prepare PBS-T by diluting 20 mL of the 10x PBS-T (K-PBST2) with 180 mL dH2O. PBS-T is stable at room temperature.
4. PBS 0.25%PS Prepare by adding 75 µL Protein Stabilizer (K-GS01) to 30 mL PBS. Vortex briefly. Leave PBS 0.25%PS at room temperature until use. Make the amount you will use for the current assay and store the remainder of the undiluted Protein Stabilizer at 4°C for future use.
5. PI(4,5)P2 Standard
   a. Prepare a 1250 pmol PI(4,5)P2 standard stock by adding 600 µL of PBS 0.25%PS to the vial of room temperature PI(4,5)P2 Standard (K-4502). Vortex for at least 1 minute to reconstitute the lipid. Spin down and leave vial at room temperature.
   b. Make seven, 5-fold serial dilutions from the 1250 pmol standard stock by adding 60 µL of previous dilution to 240 µL PBS 0.25%PS. Leave prepared PI(4,5)P2 Standards at room temperature. Store unused portion of the 1250 pmol PI(4,5)P2 standard stock at -20°C for up to 3 months.
6. PI(4,5)P2 Extracted Samples Reconstitute the dried PI(4,5)P2 extracts with PBS 0.25%PS. We suggest adding 125 µL to 245 µL for duplicate, triplicate, or quadruplicate wells. See support protocol for extraction. Vortex samples for at least 1 minute and leave at room temperature. Spin down samples before adding to the assay plate. Stimulated cells may need higher dilutions. Dilute extraction samples as necessary.
7. PI(4,5)P2 Detector Add 1 mL of PBS 0.25%PS to 1 vial of PI(4,5)P2 Detector (K-2302) to reconstitute protein. Place vial on ice for a few minutes to allow protein to dissolve into solution. Proceed immediately to next step for Incubation Plate setup. Reconstituted PI(4,5)P2 Detector is only good for the day.
8. Incubation Plate Setup

We suggest that extractions, controls (Blank and No Lipid controls), and standards be run in duplicate or triplicate. An example to set up the PI(4,5)P2 ELISA in the incubation plate is shown below.



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Incubation Plate Setup

a. Add 60 µL/well of each standard solution (step 4.b.) in duplicate or triplicate to rows B through H of the colored incubation plate.
b. Add 60 µL/well of PBS 0.25%PS to No Lipid control wells of the incubation plate.
c. Add 120 µL/well of PBS 0.25%PS to the Blank control wells in row A of the incubation plate. (No PI(4,5)P2 detector or lipid will be added to these wells.)
d. Add 60 µL/well cell extraction samples to incubation plate. Lipid extracts should be run in duplicate, triplicate, or quadruplicate.
e. Invert the PI(4,5)P2 Detector vial (K-2302) multiple times to mix and spin down. Dilute the 1 mL of PI(4,5)P2 Detector into 6 mL of PBS 0.25% PS. Invert the tube multiple times to mix.
f. Add 60 µL/well of diluted PI(4,5)P2 detector to all No Lipid control, standard, and sample wells. Do not add detector to the 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.

9. Following incubation, transfer 100 µL from each well of the incubation plate to the corresponding well in the Detection Plate (K-4501). 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.
10. Discard the solution from the detection plate and wash the wells 3 times briefly with 200 µL/well PBS-T.
11. Secondary Detector
    a. Briefly centrifuge the vial of Secondary Detector (K-SEC2). Dilute the Secondary Detector 1:150 with PBS-T 0.25%PS. For entire plate, add 80 µL Secondary Detector and 30 µL Protein Stabilizer (K-GS01) to 12 mL PBS-T. Dilute the amount needed for the current assay. Store the remaining Secondary Detector (K-SEC2) at 4°C.
    b. Discard the last wash buffer from the detection plate. Add 100 µL of diluted Secondary Detector to each well of the plate. Seal the plate and incubate on a plate shaker at room temperature for 1 hour.
12. Discard the solution from the detection plate and wash the wells 3 times briefly with 200 µL/well PBS-T.
13. Detection
    a. Add 100 µL of TMB solution (K-TMB1) to each well. Allow color to develop for 30 minutes in dark.
    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 upon addition of stop solution. Tap the plate to mix gently. Eliminate any big air bubbles present in wells before reading the plate.
    c. Read absorbance at 450 nm on a plate reader.

Results

Cellular PI(4,5)P2 quantities can be estimated by comparing the values from the wells containing PI(4,5)P2 extraction products to the values in the standard curve. Plot the absorbance values obtained vs. amount of PI(4,5)P2 per standard to generate a standard curve. Determine where the values obtained from the PI(4,5)P2 extraction lie on the curve to obtain a measure of PI(4,5)P2 in your samples. The example standard curve was generated using non-linear regression analysis with GraphPad prism software. A sigmoidal dose-response with variable slope curve analysis (four-parameter, 4PL curve fit) was utilized.

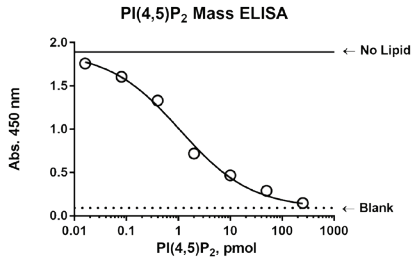

Figure 1.
PI(4,5)P2 standard curve fit using Sigmoidal dose-response (variable slope) nonlinear regression analysis. Top and Bottom of the PI(4,5)P2 standard curve have been constrained to No Lipid control and Blank control, respectively.

Support Protocol: Lipid Extraction

PI(4,5)P2 extraction protocol as verified with 1 x 10^6 NIH-3T3 mouse fibroblast cells grown to 80% confluency per well in a 6-well or a 12-well plate. Larger or smaller amounts of cells may require proportional adjustments in volume. The amount of cells necessary for PI(4,5)P2 quantification needs to be determined for each cell type.

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 (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(4,5)P2 from cells

1. Collect Cells
   For adherent cells, remove media by gentle aspiration and immediately add 1 mL ice cold 0.5 M TCA. For non-adherent cells, spin the cells down, decant media, then add 1 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 2 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.

2. Wash Pellet
   Add 1 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.

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3. Extract neutral lipids
   Add 1 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 750 µL 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 2 mL centrifuge tube. Discard the pellet.

5. Phase split
   To supernatant from step 4, add 250 µL of CHCl3 (avoid using plastic pipette tip) and 450 µL of 0.1 NHCl. 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 0.5 mL of the organic (lower) phase, preferably with a positive displacement pipette, into a clean 1.5 mL vial and dry in a vacuum dryer (45 – 60 min). Dried lipid can be stored at -20°C for up to 1 year. The dried lipid should not be visible. If there is a visible substance at the end of this step, it is most likely cell debris that was not eliminated in the extraction. Do not attempt to dissolve the cell debris in PBS 0.25%PS. This black to yellow substance should also be avoided when pipetting the lipid extraction samples into the PI(4,5)P2 Mass ELISA wells.

References

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Related Products

Products	Catalog Number
Other PIP Mass ELISAs	
PIP_3 Mass ELISA Kit	K-2500s
PI(3)P Mass ELISA Kit	K-3300
PI(3,4)P_2 Mass ELISA Kit	K-3800
PI(4)P Mass ELISA Kit	K-4000E
Activity Assays	
PI3-Kinase Activity ELISA	K-1000s
PTEN Activity ELISA	K-4700
PI(4)P 5-Kinase Activity Assay	K-5700
PI(4,5)P2 Binding Proteins and Antibodies	
PI(4,5)P2 Grip	G-4501
Purified Anti-PtdIns(4,5)P2 IgM	Z-P045

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