Version 29-06/25

User's Manual

PI(3)P ELISA Kit

REF **DEIA-XYZ13**

Σ **96T**

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This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

The Phosphatidylinositol 3-phosphate ELISA Kit measures the amount of PI(3)P extracted from cells by means of a competitive ELISA.

General Description

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.

Principles of Testing

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.

Please read this entire section and the assay notes section before beginning the assay. Prior to use, place **PI(3)P Detector** and **Secondary Detector** on ice and bring all other kit components and extracted PI(3)P samples to room temperature.

Reagents And Materials Provided

1. Coated Strip-well Detection Plate, 1 plate
2. PI(3)P Standard, 12.2 µg
3. PI(3)P Detector, 2 vials
4. Secondary Detector, 300 µL
5. 10× PBS-T, 20 mL
6. Protein Stabilizer, 2×600 µL
7. TMB Solution, 12 mL
8. 1 N H_2SO_4 Stop Solution, 10 mL
9. Colored 96-well polypropylene U-bottom plate, 1 plate
10. Plate sealers, 2 seals

Materials Required But Not Supplied

1. Extracted PI(3)P samples (See PI(3)P Extraction Protocol at the end of this document)
2. Buffers and solvents for PI(3)P extractions: Trichloroacetic Acid, EDTA, Methanol, Chloroform, and 12 N

HCl

3. Vacuum dryer
4. 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.

Reagent Preparation

1. PBS-T Buffer: Prepare by adding the entire bottle of **10× PBS-T Buffer 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 µL Protein Stabilizer 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.

Assay Procedure

1. PI(3)P Standard

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**. Vortex for at least 1 minute to rehydrate the lipid. Spin down and leave vial at room temperature before use.

Make five, 4-fold serial dilutions from the 400 pmol standard stock by adding 100 µL of the 400 pmol PI(3)P standard stock or previous dilution to 300 µL 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 µL to 245 µL for duplicate, tripli-cate,

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** 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 µL/well of each standard solution (400 pmol through 0.39 pmol) in triplicate or duplicate to rows B

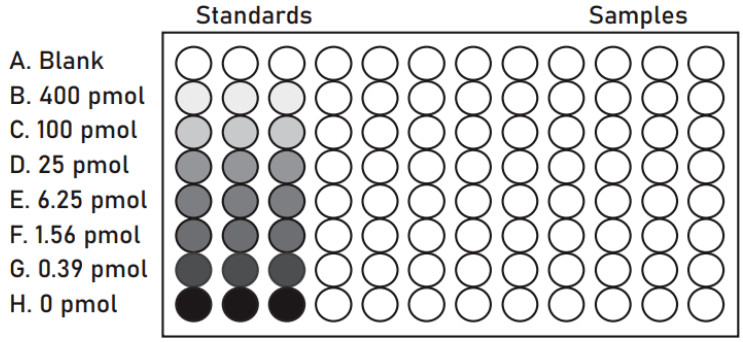
through G of the Incubation Plate (colored plate).

b. Add 60 µL/well of PBS-T 3%PS to the 0 pmol control wells in row H of Incubation Plate.

c. 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.)

d. Add 60 µL/well cell extraction samples to Incubation Plate. Lipid extracts should be run in duplicate or triplicate.

e. 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.



5. Following the incubation, mix and transfer 100 µL from each well to the corresponding well in the Detection Plate. 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

6. Wash the Detection Plate 3 times with 200 µL/well PBS-T

7. Secondary Detector

Briefly centrifuge the vial of **Secondary Detector**. Dilute the Secondary Detector 1:100 with fresh PBS-T 3%PS (Add 120 µL **Secondary Detector** and 360 µL **Protein Stabilizer** 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.

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

9. Detection

Add 100 µL of TMB solution 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 H_2SO_4 Stop Solution 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.

10. Read absorbance at 450 nm on a plate reader.

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 adjust-ments

of volumes. If you have never run lipid extractions or have little experience with the reagents listed below, please contact Creative-diagnostics.

A. Solutions for Extraction

1. 0.5 M TCA For 50 mL, dissolve 4.08 g TCA (Trichloroacetic Acid) in dH_2O and bring volume to 50 mL.
2. 5% TCA with 1 mM EDTA For 50 mL, dissolve 2.5 g TCA in dH_2O, add 100 µL 0.5 M EDTA, and bring volume to 50 mL with dH_2O.
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 dH_2O.

B. Extraction of PI(3)P from cells

1. Collect Cells

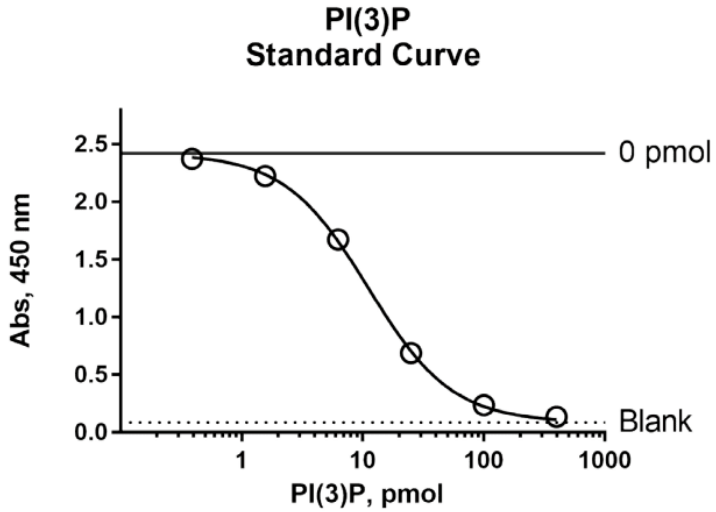
   a. For adherent cells in a 75 cm^2 flask, remove medium by gentle aspiration and immediately add 5 mL ice cold 0.5 M TCA. Incubate cells on ice for 5 minutes. Scrape the cells from flask with additional 0.5 M TCA if needed and transfer to a 15 mL centrifuge tube. Centrifuge at 3000 RPM (approximately 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 superna-tant. 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.

Calculation

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.



Detection Range

400 - 0.39 pmol PI(3)P

Precautions

Disclaimer

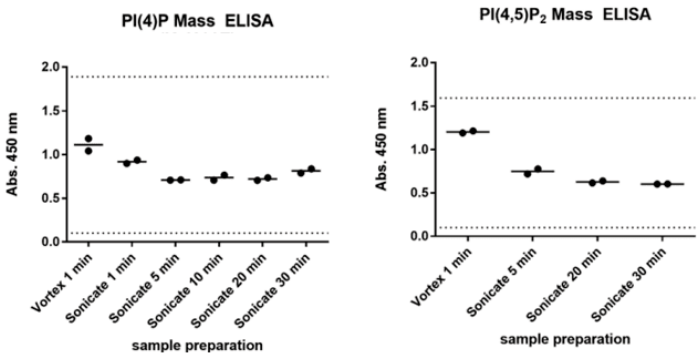
The PIP Mass Assays are used to quantify the total amount of the specific lipid extracted from cells. However, since the lipids are substrates for enzymes in multiple pathways the data obtained may not correlate with what has been observed with isolated enzyme reactions or visualized with immunohistochemistry

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. It can be difficult to reproduce conditions of sonication (examples are shown in the garphs 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 inconstancies in your data if it is not held constant.



5. 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.
6. 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.