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Technical Data Sheet

For research use only
*Not intended or approved for
diagnostic or therapeutic use.*

PIP₃ Mass ELISA Kit™

Product Number: K-2500

Kit Includes:

1 Incubation plate: 96-well polypropylene U-bottom plate
2 vial PI(3,4,5)P₃ standard
1 vial PI(3,4,5)P₃ Detector
1 Detection plate: PI(3,4,5)P₃ coated flat-bottom clear 96-well plate (twelve 8-well strips), pre-blocked.
1 vial Secondary Detector
1 bottle TMB solution

Researcher provides:

Buffers and solvents for extractions, incubations, and washes
Acetate plate sealers/parafilm
Absorbance plate reader
Source of PI(3,4,5)P₃ extracts
Stop solution for TMB development

Background

The production of PI(3,4,5)P₃ from PI(4,5)P₂ by type-1 PI 3-kinases (PI3-K) is important in multiple cell signaling pathways. Typically, experiments to measure PI 3-K activity have involved phosphorylation of a phosphoinositide substrate using ³²P, then extraction of radioactive products, and separation using thin-layer chromatography. The assay plate method developed by Echelon Biosciences, Inc. allows the user to determine PI 3-K activity by measuring the amount of PI(3,4,5)P₃ extracted from cells by means of standard ELISA format, eliminating the need for radioactivity, organic solvents, and thin layer chromatography.

The assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(3,4,5)P₃ produced. Once PI(3,4,5)P₃ has been extracted from cells samples, it is first incubated with a PI(3,4,5)P₃ detector protein, then added to the PI(3,4,5)P₃-coated microplate for competitive binding. A peroxidase-linked secondary detection reagent and colorimetric substrate is used to detect PI(3,4,5)P₃ detector protein binding to the plate. The colorimetric signal is inversely proportional to the amount of PI(3,4,5)P₃ extracted from cells. The assay is sensitive to 1 pmol PIP₃, and requires approximately ~3 x 10⁶ cells per data point depending on the experimental system used.

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TDS K-2500 Rev: 1 (11/07/05)

Basic Protocol:

Buffer Preparation

PI(3,4,5)P₃ Buffer

50 mM Hepes
150 mM NaCl
1.5% Na Cholate
pH 7.4

TBS

150 mM NaCl
10 mM Tris
pH 7.5

TBS-Tween

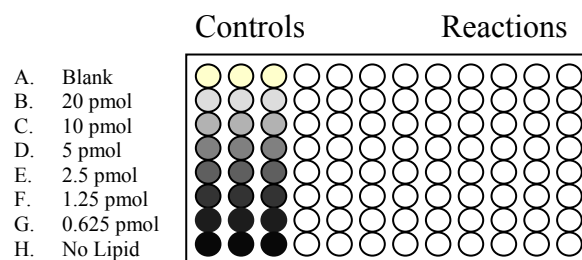
TBS + 0.05% Tween-20

Stop Solution for ELISA Color Development

0.5M H₂SO₄

Incubation:

Stock solution of PI(3,4,5)P ₃	pmol/50 μ L well	μ L standard	μ L PIP ₃ buffer
0.4 μ M	20	60	0
0.2 μ M	10	30	30
0.1 μ M	5	15	45
0.05 μ M	2.5	7.5	52.5
0.025 μ M	1.25	3.75	56.25
0.0125 μ M	0.625	1.875	58.125
No Lipid control	0	0	60



Incubation Plate

1. Set up the incubation plate (colored polystyrene U-bottom micro titer plate). First, prepare the standard curve using PI(3,4,5)P₃ standard provided for this purpose. Add 400 μ L of PIP₃ buffer to vial of PIP₃ standard to prepare a 0.4 μ M stock. Vortex 1-2 minutes to resuspend the lipid.
2. We suggest that controls, standards, and samples be run in triplicate. From the 0.4 μ M stock of PI(3,4,5)P₃, make four 2-fold serial dilutions in PIP₃ buffer, as outlined in the above table. Reserve some wells for blank controls, in which secondary detection reagent alone will be added. Pipette 60 μ L of each standard dilution (in duplicate or triplicate) into the wells of the incubation plate. One suggested setup for the incubation plate is shown above. For the blank controls in row A, add 120 μ L PIP₃ buffer per well. Add 60 μ L/well of PIP₃ buffer to the no lipid controls in row H. Run lipid extracts from above protocol in triplicates. Add 60 μ L of extracts per well.
3. Dilute the PI(3,4,5)P₃ detector 1:200 with PIP₃ buffer. (Make only enough working stock of detector for use that day.) Add 60 μ L of diluted PI(3,4,5)P₃ detector to all wells except the blank controls in row A. Seal the incubation plate and incubate for 1 hour at room temperature on an orbital shaker. The plate may also be incubated

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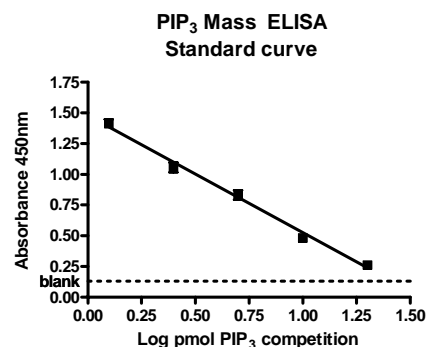
at 4 ° C overnight if desired. PI(3,4,5)P₃ detector may be stored at 4 ° C for up to one month. For longer storage, store at –20 ° C.

Detection

1. Following the incubation, transfer the reacted mixtures to the detection plate (clear flat-bottom strip plate). Transfer 100 µL from each well to the corresponding well in the detection plate. (This can easily be accomplished with a multi-channel pipettor discarding tips after each pipetting step.) Seal the plate and incubate 30-60 minutes at room temperature on an orbital shaker. Discard the solution and wash the wells 3 times with 200-300 µL TBS plus 0.05% (v/v) Tween.
2. Dilute the secondary detection reagent solution 1:40 with TBS-T. Dilute ONLY the amount you will use for the current assay and store the remainder of the secondary reagent at 4° C for future use. Add 100 µL of secondary detection reagent solution to all wells of the detection plate. Seal the plate and incubate for another 30-60 minutes on an orbital shaker. Discard the solution and wash the wells 3 times with 200-300 µL TBS-Tween.
3. Add 100 µL of TMB solution to each well. Allow color to develop for approximately 2-30 minutes. Reading the blue end product at 370 nm or 655 nm can monitor progression. (watch for color development and DO NOT overdevelop). Stop the reaction by adding 50 µL of stop solution (0.5 M H₂SO₄) to each well. Immediately read on an absorbance plate reader at 450 nm. Signal will be inaccurate 30 minutes after reaction is quenched by stop solution. (NOTE: You may not be able to accurately tell the difference between the intensities of color by eye.)

Results

Cellular PI(3,4,5)P₃ quantities can be estimated by comparing the values from the wells containing PI(3,4,5)P₃ extraction products to the values in the standard curve. Plot the absorbance values obtained vs. amount of PI(3,4,5)P₃ per standard to generate a standard curve. Determine where the values obtained from the PI(3,4,5)P₃ extraction lie on the curve to obtain a measure of PI(3,4,5)P₃ in your samples.



Storage:

Store kit at 4 ° C in a dark location. The PI(3,4,5)P₃ detector can be stored at 4 ° C and used up to one month, although signal may decrease with storage. For longer storage periods, store the detector at –20 ° C.

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Support Protocol - Extraction of PIP₃ cells

1. The procedure requires at least 5×10^6 cells to obtain sufficient PIP₃ for ELISA detection.
2. For 75 cm² flask, remove medium to the last drop by gentle aspiration, and immediately add 4 mL cold 0.5 M TCA. Incubate on ice for 5 minutes.
3. Scrape the cells from flask and transfer into a 15 mL centrifuge tube on ice. Rinse flask with 4 mL 0.5 M TCA, and add into the centrifuge tube. Centrifuge at 1500 RPM for 5 minutes at 4 °C. Discard the supernatant.
4. Add 3 mL 5% TCA/ 1 mM EDTA to the pellet. Vortex. Centrifuge at 1500 RPM for 5 minutes. Discard the supernatant. Repeat one more time.
5. Extract neutral lipids by adding 3 mL MeOH : CHCl₃ (2:1), vortex 3 times over 10 minutes at room temperature (RT), centrifuge at 1500 RPM for 5 minutes, discard the supernatant. Repeat the extraction one more time.
6. Extract acidic lipids by adding 2.25 mL MEOH : CHCl₃ : 12 M HCl (80:40:1) vortexing 4 times over 15 minutes at RT. Centrifuge at 1500 RPM for 5 minutes, transfer the supernatant to a 15 mL centrifuge tube.
7. Phase split by addition of 0.75 mL of CHCl₃ and 1.35 mL of 0.1 M HCl, vortex and centrifuge at 1500 RPM for 5 minutes to separate organic and aqueous phases. Collect organic (lower) phase, put into clean 15 mL tube, and dry in a vacuum dryer.
8. Resuspend dried lipids in 120 µL of PIP₃ Buffer (50 mM Hepes, 150 mM NaCl, 1.5% Na Cholate, pH 7.4), sonicate in a water bath for 5 minutes, leave overnight at 4 °C.

This process will allow two 60 µL samples in the PIP₃ Mass ELISA.

Solutions for Extraction of PIP₃ from cells:

0.5 M TCA

for 50 mL add 4.08 g TCA and bring to volume with H₂O.

MeOH : CHCl₃ (2:1)

For 50 mL add 33.3 mL MeOH, and 16.7 mL CHCl₃.

0.1M HCl

For 50 mL add 0.41 mL 12 M HCl to 49.39 mL H₂O.

5% TCA 1mM EDTA

For 50 mL add 18.61 mg EDTA, 2.5 g TCA to and bring to volume with H₂O.

MeOH: CHCl₃:12 M HCl (80:40:1)

For 50 mL add 33 mL MeOH, 16.5 mL CHCl₃, and 0.41 mL 12 M HCl

PIP₃ Buffer

50mM Hepes
150 mM NaCl
1.5% Na Cholate
pH 7.4

Reference:

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

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