Echelon Biosciences Inc.

PI 4-Kinase Activity ELISA Kit

K-4000K (96 tests)

Support: echelon@echelon-inc.com

Description: A competitive ELISA that detects and quantifies PI4P produced from a PI4-Kinase reaction.

Materials Provided

Catalog #	Description	Quantity
K-4005	PI(4)P Detector	0.5 µg
K-4001E	PI(4)P Detection Plate	96-well
K-4006	PI(4)P Standard	4.79 µg
K-DIL2	Diluent	1 bottle
K-4001K	PI Substrate	500 µg
K-EDTA	100 mM EDTA	500 µL
K-KBZ4	ER Buffer	1 vial
K-PBST2	10X PBS-T Buffer	20 mL
K-SEC2	Secondary Detector	300 µL
K-TMB1	TMB Solution	12 mL
K-STOPt	1 N H_2SO_4 Stop Solution	8 mL
---	96 well Colored Mixing Plate	1 plate
---	Acetate Plate Seal	3 Seals

Additional Materials Provided by User:

- Source of enzyme
- Adenosine Triphosphate (ATP)
- Absorbance microtiter plate reader capable of reading at 450 nm.

Quick Protocol

Prepare all buffers and reagents.

Set up PI4-Kinase reactions in the mixing plate. Incubate for 0.5-4 hours at 37°C or RT.

Stop kinase reactions by adding EDTA.

Set up PI(4)P Standards in the mixing plate.

Add PI(4)P Detector to the mixing plate, incubate 30 min with shaking, then transfer to the Detection Plate.

Incubate 2 hour at room temperature with shaking.

Wash the plate and add the Secondary Detector. Incubate for 1 hour with shaking.

Wash the plate and add TMB solution. Incubate for 30 minutes in the dark.

Add Stop Solution and read plate at 450 nm.

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 -80°C. Store prepared reagents as indicated in the protocol.

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Background

Phosphatidylinositol 4-phosphate (PI(4)P) is the most abundant monophosphorylated phosphoinositide found in mammalian cells[1]. It is produced by PtdIns 4-Kinases (PI4K) which phosphorylates the D-4 position of the inositol ring of PtdIns. PI 4-Kinases have been classified into two types, II and III, based on their molecular mass, and modulation by detergent and adenosine. Type II class PI 4-Kinases includes PI4KIIa (PI4K2A) which is involved in many biologic processes ranging from cell growth to endo- and exocytosis. It is also a novel regulator of tumor growth and generates PtdIns4P-rich domains within the Golgi[2]. PI4KIIb (PI4K2B) is primary cytosolic where it is recruited to the membrane and stimulates PI(4,5)P2 synthesis[3]. Type III PI 4-Kinase, PI4KIIIa (PI4K3A), plays a role in replication of the Hepatitis C virus. These viruses use PI4KIIIa to generate PI(4)P-enriched environments[4,5,6] increasing cellular levels of PI(4)P. PI4KIIIb (PI4KB), regulates the golgi reorganization during mitosis. This enzyme is essential for glucose-induced insulin secretion due to its capacity to regulate the release of secretary granules[7]. All of this makes PI 4-Kinases an interesting target for a diverse set of biological functions, disease states, and infections.

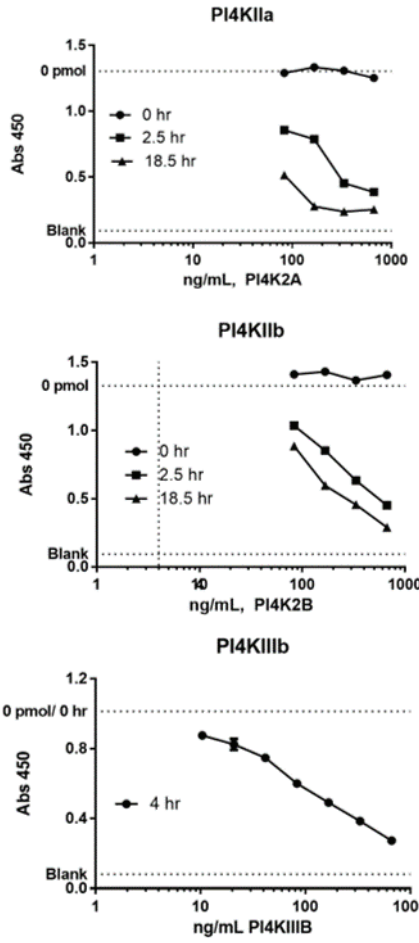
Assay Design

Echelon's PI 4-Kinase Activity ELISA has been designed to detect and quantify, the end product of a PI 4-Kinase reaction, PI(4)P. The PI 4-Kinase Activity ELISA is a competitive ELISA in which the signal is inversely proportional to the amount of PI(4)P measured. The enzyme reactions are stopped with EDTA before the PI(4)P Detector is added. This mixture is then transferred to the PI(4)P Detection Plate for competitive binding. A peroxidase-linked Secondary Detector and colorimetric detection is used to detect the amount of PI(4)P Detector binding to the plate.

Assay Notes

1. The incubation steps for this assay require a plate shaker. If a plate shaker is unavailable the incubation steps can be ran for two hours without shaking. A reduction in signal and some loss in sensitivity may be observed.
2. The PI(4)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 PI(4)P 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. The assay has been tested with PI4KIIA (cat# P21-10G), PI4KIIB (cat# P22-10G), and PI4KIIIB (cat# P32-102G) and PI4KIIB that was immuno-precipitated from HepG2 cells using an antibody from Bethyl Labs (cat# A303-734A). See Figure 1 for reaction conditions used.
5. ATP does not interfere in the assay at 3,000 µM or lower. Higher concentrations have not been tested. The Km of ATP for PI4KIIIB (Cat # P32-10G) was determined to be 92.7 µM. Literature = ~ 127 based on 3 publications. Optimization of ATP concentration may be needed depending on your enzyme. Type III PI4Ks are inhibited by high doses of adenosine (>1mM) and type II PI4Ks are sensitive to low doses of adenosine.
6. Buffers not provided in the kit may cause interference in the assay. If, for example, your enzyme storage buffer is causing interference, the enzyme or the buffer should be added to the standards and controls. The enzyme can be deactivated by adding the 4 mM EDTA solution or the prepared PI(4)P standard to the well before the enzyme is added.
7. The 4 mM EDTA solution is an enzyme stop solution.

Figure 1



The PI(4)P product of recombinant full-lenth human PI4KIIa enzyme (cat# P21-10G) was measured in the PI 4-Kinase Activity ELISA (cat# K-4000K). The enzyme reactions were ran for 0, 2.5 and 18.5 hrs at 37°C with shaking. ATP and PI Substrate were held constant at 100 mM and 222.2 mg/mL respectively. The specific activity of PI4KIIa was calculated at 155.1 - 369.6 depending on conditions used.

The PI(4)P product of recombinant full-lenth human PI4KIIb enzyme (cat# P22-10G) was measured in the PI 4-Kinase Activity ELISA (cat# K-4000K). The enzyme reactions were ran for 0, 2.5 and 18.5 hrs at 37°C with shaking. ATP and PI Substrate were held constant at 100 mM and 222.2 mm/mL respectively. The specific activity of PI4KIIb was calculated at 301.2 - 991.5 depending on conditions used.

The PI(4)P product of recombinant full-lenth human PI4KIIIb enzyme (cat# P32-10G) was measured in the PI 4-Kinase Activity ELISA (cat# K-4000K). The enzyme reactions were ran for 0 and 4 hrs at room temperature with shaking. ATP and PI Substrate were held constant at 1,111 mM and 333.3 mg/mL respectively. The specific activity of PI4KIIIb was calculated at 55.5 - 88.6 depending on

8. DTT can interfere with the enzyme detection/reaction. If this is used in the assay a control should be run to determine the degree of interference.
9. The PI Substrate (K-4001K) is provided as mg/mL concentration. The substrate contains multiple PI substrates of different chain length and size. The average molecular weight is 895 g/mol.
10. The amount of enzyme to use per PI4K reaction will vary depending on your individual experiment, enzyme, and the cell type you are using. Whether you are using purified PI4K or enzyme from immunoprecipitated from cell lysate, it is recommended to run an enzyme titration first to determine the optimum amount of enzyme to use. When using purified recombinant human PI4K's from Echelon (cat # P21-10G, P22-10G, or P32-10G) a good starting point is 1,000 ng/mL.
11. A "No enzyme", "No PI substrate", and "Time = 0" (with all components of the enzyme reaction but stopped with 4 mM EDTA) are suggested as controls for your enzyme reactions.
12. Longer incubation times (> 4 hr), for the enzyme reaction, can be used for samples with low concentrations of enzyme. The duration of your enzyme reaction should be optimized using your sample.

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Protocol for Determination of PI4-Kinase Activity

Please read this entire section and the Assay Notes section before beginning the assay.

1. Place buffers (K-PBST2, K-DIL2, K-KBZ4), 100 mM EDTA (K-EDTA), PI(4)P Standard (K-4006), PI Substrate (K-4001K), TMB Solution (K-TMB1), 1 N H_2SO_4 Stop Solution (K-STOPt), and Colored Mixing Plate at room temperature. Place PI(4)P Detector (K-4005), Detection Plate (K-4001E), and Secondary Detector (K-SEC2) on ice (or 4°C) until use.
2. Prepare 1X PBS-T buffer by adding 20 mL of 10X PBS-T Buffer (K-PBST2) to 180 mL dH_2O. Keep at room temperature.
3. Prepare 4 mM EDTA by diluting 180 µL of 100 mM EDTA (K-EDTA) in 4.32 mL 1X PBS-T (step 2). Keep at room temperature.
4. Prepare Diluent by adding 12 mL dH_2O to the bottle of Diluent (K-DIL2). Vortex. Let sit 5 min. Vortex. Keep at room temperature during use. Store long term at -20°C.
5. Prepare 0.6 mL 3X ER Buffer by adding dH_2O and desired amount of ATP directly to the vial of ER Buffer (K-KBZ4). Eg. 400 µL dH_2O and 200 µL of 10 mM ATP. Vortex to mix. Keep at room temperature during use. Add 5 µL 3X ER Buffer to all wells of the Colored Mixing Plate. Once the ER Buffer is brought up into solution, it can be stored long term, without ATP, at -20°C. Do not add ATP until day of use.
6. The PI Substrate (K-4001K) must be at room temperature before use. Allow to stand at room temperature for 15-60 minutes then add 150 µL dH_2O to the PI substrate. Vortex at medium speed for 1 minute. Then add 350 µL dH_2O for a final concentration of 1 mg/mL. Keep at room temperature during use. Add 5 µL prepared PI Substrate to all wells of the Colored Mixing Plate. If multiple days of use are needed, add 500 µL Chloroform to dried PI Substrate (K-4001K) vortex and aliquot into desired sizes. Dry in speedvac and store at -20°C. Adjust protocol accordingly when using alternate PI Substrate sizes in assay.
7. Add 5 µL of Diluent (step 4) to all wells of the Colored Mixing Plate except the enzyme reaction wells.
8. Prepare your PI 4-Kinase in Diluent (step 4). Add 5 µL PI 4-Kinase preparations to the enzyme reaction wells of the Colored Mixing Plate. Incubate the Colored Mixing Plate for 0.5-4 hours at room temperature to 37°C with or without moderate shaking. Enzyme reactions will be chemically stopped, with EDTA, at step 11a.
9. The PI(4)P Standard (K-4006) must be at room temperature before use. Allow vial to stand at room temperature for 15-60 minutes then add 300 µL of 4 mM EDTA (step 3). Vortex at medium speed for 1 minute. This is the 500 pmol PI(4)P Standard. The 500 pmol PI(4)P Standard can be stored for up to a week at -20°C.
10. Serial dilute the 500 pmol PI(4)P Standard (step 9) 4 fold, 5 times, in 4 mM EDTA (step 3) Example: 50 µL previous dilution + 150 µL 4 mM EDTA. Keep standard set at room temperature . To
11. Colored Mixing Plate:
    a. Add 30 µL / well of 4 mM EDTA (step 3) to enzyme reaction wells, 0 pmol wells, and Blank controls. Do not add 4 mM EDTA to the PI(4)P standard wells. This is the enzyme stop for the enzyme reactions.
    b. Add 30 µL / well of each PI(4)P standard (steps 9-10) to the Colored Mixing Plate according to the plate layout. All wells of the colored Mixing plate should have 45 µL / well.
12. Prepare 10 µg/mL PI(4)P Detector Stock by adding 100 µL dH_2O to the vial of lyophilized PI(4)P Detector (K-4005). Hand mix then place on ice for at least 1 minute. Hand mix again before use. Store 10 µg/mL PI(4)P Detector long term at -20°C.
    a. Add 90 µL of the 10 µg/mL PI(4)P Detector to 8 mL Diluent. Keep at room temperature.
    b. Add 75 µL/ well Diluent (step 4) to the blank controls.
    c. Add 75 µL/well prepared PI(4)P Detector (step 12a) to all wells of the colored mixing plate except blank controls. All wells of the mixing plate should have 120 µL/ well. Cover and incubate on plate shaker for 30 minutes. Place the Detector Plate (K-4001E) at room temperature.
13. Mix each well with pipette then transfer 100 µL from each well of Colored Mixing Plate to corresponding well of the PI(4)P Detection Plate (K-4001E). Cover and incubate on plate shaker for 2 hour. A shorter incubation can be used but will produce a lower signal.
14. Discard solution in PI(4)P Detection Plate then wash 3 times with 200 µL /well 1X PBS-T Buffer (step 2). Leave last wash in plate and proceed to next step.
15. Dilute 270 µL of Secondary Detector (K-SEC2) with 3 mL Diluent (step 4). Then add 9 mL 1X PBS-T Buffer (step 2). Mix by inverting tube. Discard solution in PI(4)P Detection Plate then add 100 µL/ well prepared Secondary Detector. Cover and incubate on plate shaker for 1 hour.

Table 1, PI(4)P Suggested Plate Layout

	Standard and Controls			Enzyme Reactions (ER)								
	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	ER1	ER1	ER1	ER10	ER10	ER10	ER18	ER18	ER18
B	500 pmol	500 pmol	500 pmol	ER2	ER2	ER2	ER11	ER11	ER11	ER19	ER19	ER19
C	125 pmol	125 pmol	125 pmol	ER3	ER3	ER3	ER12	ER12	ER12	ER20	ER20	ER20
D	31.25 pmol	31.25 pmol	31.25 pmol	ER4	ER4	ER4	ER13	ER13	ER13	ER21	ER21	ER21
E	7.81 pmol	7.81 pmol	7.81 pmol	ER5	ER5	ER5	ER14	ER14	ER14	ER22	ER22	ER22
F	1.95 pmol	1.95 pmol	1.95 pmol	ER6	ER6	ER6	ER15	ER15	ER15	ER23	ER23	ER23
G	0.49 pmol	0.49 pmol	0.49 pmol	ER7	ER7	ER7	ER16	ER16	ER16	ER24	ER24	ER24
H	0 pmol	0 pmol	0 pmol	ER9	ER9	ER9	ER17	ER17	ER17	ER25	ER25	ER25

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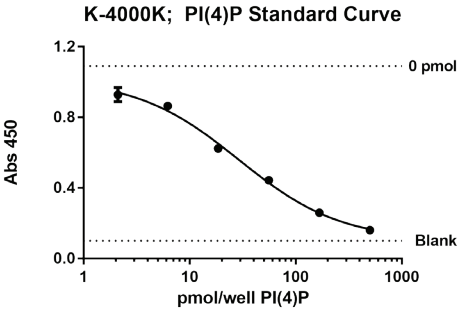
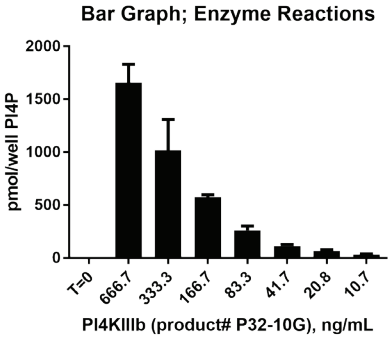
16. Discard solution in PI(4)P Detection Plate then wash 3 times with 200 µL /well 1X PBS-T Buffer (step 2).
17. Add 100 µL/well TMB Solution (K-TMB1). Develop for 30 minutes in a dark location.
18. Stop reaction by adding 50 µL/well Stop Solution (K-STOPt) and read absorbance 450 nm on plate reader.

Results

PI 4-Kinase activity can be estimated by comparing the absorbance values from the wells containing enzyme reaction products to the values in the standard curve. Plot the absorbance values obtained vs. log of PI(4)P in pmol per standard to generate a standard curve using sigmoidal dose-response (variable slope) correlation. Determine the PI(4)P level in pmol by interpolation from absorbance values obtained from the enzyme reactions. See figures below.



PI(4)P Standard Curve was generated using non-linear regression analysis with GraphPad software. A log(agonist) vs. response -- Variable slope (four parameters) analysis was utilized. For best results, constrain standard top and bottom using the 0 pmol PI(4)P and Blank control.



Reactions ran at room temperature for 3 hours with shaking with 0.33 mg/mL PI Substrate (K-4001K) and 1.11 mM ATP.

Support Protocol

Immunoprecipitation of PI4KB From Cells

The following support protocol for immunoprecipitation of PI4K from cells has been validated for use with the PI 4-Kinase Activity Assay. Further optimization of this protocol and/or different IP protocols can be used to IP PI4K from cells depending on the needs and experience of the user.

Materials Needed	Company	Catalog Number
1.5 mL Centifuge Tubes	N/A	N/A
Lysis Buffer (25 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 1mM EDTA, 5% Glycerol)	N/A	N/A
Protease Inhibitor Cocktail	Sigma	P8340
Protein A Agarose Beads	Pierce	20333
PI4KB Antibody	Bethyl Labs	A303-734A
Diluent	Echelon	K-DIL2
PBS	N/A	N/A

This protocol was tested with an estimated 7 X 106 HepG2 cells. To maximize PI4K activity; keep all solutions ice cold, carry out all reactions on ice or at 4°C and use a centrifuge that is equilibrated at 4°C. It is suggested to run controls such as a "bead/PI4K Antibody" with no cell lysate or a "Bead only" with cell lysate and no PI4K Antibody.

Reagent Preparation

Lysis Buffer: Prepare the Lysis Buffer according to the recipe listed on the materials. Add the protease inhibitor cocktail fresh at a 1:100 dilution. Place buffer on ice and chill until ice cold.

Cell Lysis

1. Place cell culture dish on ice and wash cells twice with ice cold PBS.
2. Add 0.5 mL of ice cold Lysis Buffer to cells. Scrape cells and transfer mixture into a cooled 1.5 mL centrifuge tube.
3. Incubate cells for 15 minutes with constant agitation at 4 C.
4. Centrifuge cells for 10 minutes at 14,000 x g to pellet cells.
5. Transfer supernatant to a fresh, cooled 1.5 mL centrifuge tube and place on ice. Discard pellet.

Immunoprecipitation

1. Transfer 400 µL of the supernatent to a fresh, cold, 1.5 mL centrifuge tube.
2. Add 2 µL of the PI4K Antibody (Bethyl Labs) to the lysate. Incubate 3 hours to overnight at 4°Cwith agitation.
3. Add 60 µL of the 50% Protein A Agarose Beads to the mixture and incubate 2-3 hours at 4°C.
4. Briefly centrifuge to pellet beads. Discard the supernatant.
5. Wash the bead complex three times with ice cold PBS. Centrifuge and discard solution after each wash.
6. Wash 1-3 times with Diluent.
7. Resuspend bead complex in minimum (~ 30 µL) Diluent (for ~50% Protein A Agarose Beads).
8. Proceed immediately with the PI 4-Kinase Activity Assay by adding 5µL / well IP enzyme (with bead) to the Colored Mixing plate. See the "PI 4-Kinase Activity Assay Protocol" section in the main protocol.

References

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References (Background)

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Related Products

Products	Catalog Number
PIP Mass Assay	
PI(4)P Mass ELISA Kit	K-4000E
PI4K Inhibitor	
PIK-93 (PI4KIIIb inhibitor)	B-0306
PI 4-Kinases	
PI4K2A (PI4IIKa), active	P21-10G
PI4K2B (PI4KIIb), active	P22-10G
PI4KB (PI4KIIIb), active	P32-102G
Lipid Antibodies and binding proteins	
PI(4)P Grip (SidC-3C)	G-0402
Purified Anti-PI(4)P Antibody IgM	Z-P004

Please visit our website at www.echelon-inc.com for more phospholipid products.

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