Echelon Biosciences Inc.

Class III PI3-Kinase Kit

K-3000 (96 tests)

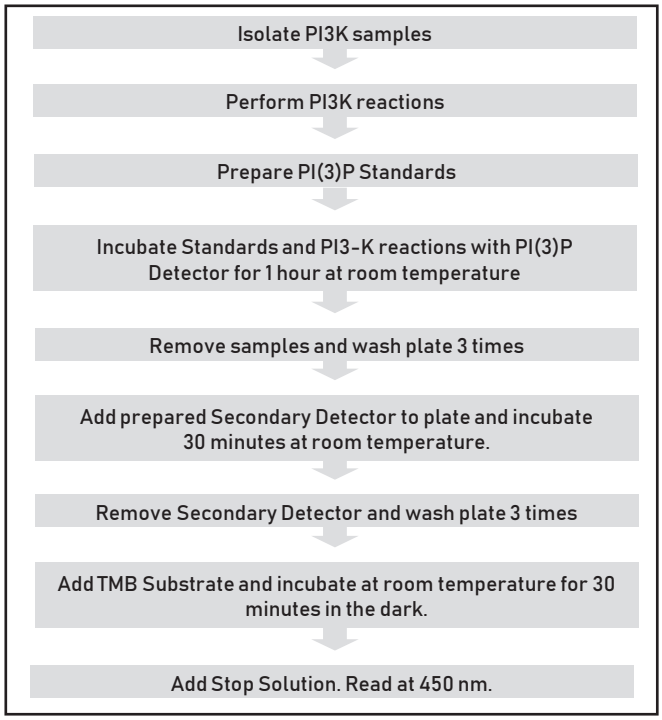
Support: echelon@echelon-inc.com

Description: 96-well ELISA Assay for Detection of PI(3)P

Materials Provided

Catalog #	Description	Quantity
K-3001	PI(3)P Detection Plate, 12 x 8-strip well	1 plate
K-3002	PI Substrate, diC_8, MW = 608.6 g/mol	2 vials
K-3003	PI(3)P Standard, diC_8, MW = 732.5 g/mol	1 vial
K-3004	5x PI(3)P Detection Buffer (5x DB)	1 bottle
K-EDTA	100 mM EDTA, pH 8	1 vial
K-TBST	10x TBS-T Buffer	1 bottle
K-3305	PI(3)P Detector	2 vials
K-DIL3	5x Diluent	1 vial
K-SEC2	Secondary Detector	1 vial
K-TMB1	TMB Solution	1 bottle
K-STOPt	1 N H_2SO_4 Stop Solution	1 bottle
---	Colored 96-well U-bottom plate	1 plate
---	Clear acetate sheet, 1 side adhesive	3 sheets

Quick Protocol



Additional Materials Provided by User:

- PI3-Kinase enzyme
- 2x Kinase reaction buffer (see Assay Note 1)
- Absorbance plate reader capable of reading at 450 nm
- Plate shaker

Storage: Store kit part 1 at 4°C. Store kit part 2 at -20°C.

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Background

PI3-Kinases (PI3-K) are grouped into three classes according to their structural homology and in vivo lipid substrate preference. Class III PI3-K uses phosphatidylinositol (PI) as substrate to generate PI(3)P product. While Class I PI3-Ks are well-recognized for their role in cell growth and division, the physiological roles of the Class II and III enzymes are still emerging.[1] Typically, experiments to measure PI3-K activity have involved phosphorylation of a phosphoinositide substrate using ^{32}P, then extraction of radioactive products, and separation using thin-layer chromatography or HPLC. The assay plate method developed by Echelon Biosciences allows the user to determine PI3-K activity, using either recombinant or immunoprecipitated enzyme, in a standard ELISA format, eliminating the need for radioactivity, and thin layer chromatography or HPLC.

Assay Design

Echelon's Class III PI3K Activity Assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(3)P produced. After the PI3-K reactions are complete and quenched, reaction products are diluted and added to the PI(3)P-coated microplate, for competitive binding to a PI(3)P detector protein. The amount of PI(3)P detector bound to the plate is determined through colorimetric detection. This ELISA is specific to PI(3)P detection. As low as 1 pmol PI(3)P can be detected in a 100 µL detection mixture, corresponding to a sensitivity of 10 nM.

Assay Notes

1. The composition of the 2x kinase reaction buffer can be determined by the user. Two sample recipes for 2x kinase reaction buffer are listed below for human Vps34 (PIK3C3). For best result, prepare the 2x kinase reaction buffer freshly on day of assay. To offset any potential interference caused by the kinase reaction buffer to the detection of PI(3)P product, make sure to appropriately dilute PI(3)P standards with Detection Buffer Blend as shown in assay protocol.
   a. Recipe #1: 100 mM HEPES pH 7.5, 300 mM NaCl, and 2 mM CHAPS, 10 mM MnCl2, 2 mM DTT and 100 µM ATP.
   b. Recipe #2: 20 mM Tris pH 8, 200 mM NaCl, 2 mM EDTA, 20 mM MnCl2, and 100 µM ATP.
2. User should optimize the amount of PI3-Kinase in each reaction by doing an enzyme titration. We suggest 5 nM PI3-K enzyme as a starting point for optimization. PI Substrate titration is optional. Increase the PI Substrate if low or no PI(3)P product is detected.
3. We also suggest including one reaction without PI3-K enzyme as a "No Enzyme" control; and one reaction without PI as a "No Substrate" control.
4. It's important to dilute PI3-K reaction 8-fold into 2x PI(3)P detection buffer. Too high a strength of the reaction buffer in detection mixture will interfere with the PI(3)P detection.
5. Strip wells not in use should be removed from the plate, sealed in a plastic bag, and stored at 4°C for later use.
6. Never let the detection plate dry out after the assay has started. Always prepare the next solution before discarding the current one from the detection plate.

Reagent Preparation

Place a vial of PI(3)P Detector (K-3305) and the vial of Secondary Detector (K-SEC2) on ice. Bring PI Substrate (K-3002), PI(3)P Standard (K-3003), Detection Plate (K-3001) and other reagents to room temperature prior to use.

Prepare 2 mL Detection Buffer Blend (DBB) by adding 800 µL of 5x PI(3)P Detection Buffer (K-3004), 50 µL of 100 mM EDTA, and 125 µL of 2x Kinase Reaction Buffer to 1,025 µL ddH2O.

Dilute 20 mL 10x TBS-T buffer (K-TBST) with 180 mL ddH2O for 200 mL TBS-T buffer.

Prepare 1x Diluent for PI(3)P Detector reconstitution and dilution. Prepare only the volume needed for the day of assay. For using the whole Detection Plate, dilute 1.4 mL of 5x Diluent (K-DIL3) with 5.6 mL ddH2O. Leave 1x Diluent at room temperature prior to use.

Assay Protocol

Please read this entire section and the assay notes section before beginning the assay.

Kinase Reaction

1. Isolate or prepare PI3-Kinase according to user's standard protocol. For human cells and tissue samples, it's recommended to use the anti-hVps34 antibody (cat# Z-R015) to immunoprecipitate human Vps34 enzyme. Please refer to Z-R015 Technical Data Sheet (TDS) for the IP protocol.
2. Prepare a 500 µM PI stock by adding 99 µL of ddH2O to a vial of 30 µg PI Substrate. Vortex the vial at maximum speed for 30 seconds to fully reconstitute the lipid. Spin down briefly and leave vial at room temperature. After use, store PI stock solution at -20°C for up to 3 months.
3. We suggest the following for setting up PI3-K reactions in the colored incubation plate:
   For each PI3-K reaction, add the following to a single well of the 96-well incubation plate: 4 µL of 500 µM PI substrate (2 nmol, see assay note 2), 12.5 µL of 2x kinase reaction buffer, enough ddH2O to bring the total volume (including PI3-Kinase) to 25 µL, and finally add PI3-Kinase to start the reaction. Seal the plate with plate sealer.
   If using immunoprecipitated enzyme bound to beads (in a centrifuge tube), add 12.5 µL of 2x kinase reaction buffer to the beads, 8.5 µL ddH2O, then add 4 µL of 500 µM PI substrate to start the reaction. Scale up the reaction volume to cover all the beads if needed.
4. Let the kinase reaction proceed for appropriate time (to be determined by user, typically 0.5-3 hours) at desired temperature: room temperature, 30°C or 37°C. Do not shake vigorously.
5. Quench the kinase reactions by adding 5 µL of 100 mM EDTA (K-EDTA) to each well of 25 µL reaction. Then dilute each quenched reaction with 90 µL ddH2O. If enzyme is bound to beads or otherwise immobilized and the reaction is set up in a centrifuge tube, the quenched enzyme reaction (120 µL) can then be separated from the beads by centrifugation and transferred to the colored incubation plate for ELISA or to a new centrifuge tube for storage at –20°C.
6. Add 80 µL of 5x PI(3)P Detection Buffer (K-3004) to each 120 µL reaction mixture to bring the final volume to 200 µL ([PI] + [PI(3)P] = 10 µM total in Detection Buffer Blend). This will provide enough sample for triplicate assay points in following ELISA detection step.

Incubation and Detection (ELISA)

1. Prepare PI(3)P Standard solutions in DBB.
   a. Add 410 µL of DBB to the vial of PI(3)P Standard (K-3003) for a concentration of 10 µM. Vortex at maximum speed for 30 seconds to fully reconstitute the lipid. Spin down brief-

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ly and leave vial at room temperature.

b. Further dilute 10 µM PI(3)P 4-fold serially in DBB into unused wells of the incubation plate or micro tubes for concentrations of 2.5 µM through 9.8 nM, respectively. Refer to Table 1 below to make enough for triplicate wells of standard curve at 50 µL/well. Store unused portion of 10 µM PI(3)P at -20°C for up to 2 weeks.

2. Each vial of PI(3)P Detector (K-3305) provides enough protein for the entire 96-well detection plate. Reconstitute PI(3)P detector by adding 200 µL 1x Diluent to a vial that will be used on the day of assay. Leave vial on ice to allow the lyophilized detector to reconstitute for a few minutes before use in step 4. Proceed immediately to next step.
3. Set up the Detection Plate (K-3001). An example of a triplicate assay is shown in the detection plate layout in Table 2 below.
   a. Add 50 µL of DBB and 50 µL 1x Diluent to Blank control wells H1-H3.
   b. Add 50 µL DBB to wells G1-G3 as No Lipid control (NL).
   c. Add 50 µL PI(3)P standard dilutions (step 1) to wells 1-3 of rows A through F as indicated.
   d. Add 50 µL of each PI3-K reaction mixture from incubation plate (or centrifuge tube) to triplicate wells as indicated.
4. Incubate with PI(3)P Detector
   a. Invert the vial of reconstituted PI(3)P detector (from step 2) 10 times to mix gently and spin down. Pipet up and down multiple times and dilute 31-fold into 1x Diluent. For entire K-3001 Detection Plate, dilute 190 µL of the detector stock solution with 5.7 mL of 1x Diluent. Mix well by inverting the tube 10 times. Do not vortex PI(3)P Detector.
   b. Add 50 µL of diluted PI(3)P detector to all wells of the detection plate in use except blank control wells H1-H3.
   c. Seal plate and incubate on a plate shaker (250-300 rpm) at room temperature for exactly 1 hour. Caution: Prolonged incubation time will reduce the assay signal strength.
5. Discard solution from Detection Plate and wash plate 3 times with TBS-T 200 µL/well.
6. Dilute Secondary Detector (K-SEC2) 1:200 with TBS-T. Prepare enough for current assay only. For entire plate, dilute 60 µL Secondary Detector with 12 mL TBS-T and mix gently by inverting the tube 10 times. Discard the last TBS-T wash from plate, and add 100 µL diluted Secondary Detector to each well. Seal plate and incubate for 30 minutes at room temperature on a plate shaker.
7. Discard solution from Detection Plate and wash plate 3 times with TBS-T 200 µL/well.
8. Discard TBS-T wash completely from plate. Add TMB Solution (K-TMB1, room temperature) 100 µL to each well. Seal plate with a new sealer. 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 (K-STOPt) 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.
   Use caution when handling corrosive acid stop solution.
9. Read absorbance at 450 nm on a plate reader.

Data Analysis

Enzyme activity can be estimated by comparing the values from the wells containing enzymatic reaction products to the values in the PI(3)P standard curve.

Plot the absorbance values (OD) vs. Log PI(3)P in pmol to generate a standard curve using Sigmoidal dose-response (variable slope), non-linear regression analysis as shown below. It is recommended to constrain the curve Top to average OD of No Lipid controls and curve bottom to average OD of the Blank controls. Determine the PI(3)P value in pmol for each enzymatic reaction assay point (50 µL out of 200 µL final reaction mixture) by interpolation from the standard curve. PI(3)P percentage conversion from 500 pmol initial

Table 1. PI(3)P Standards

[PI(3)P]	Dilution Factor	PI(3)P , Volume and Source	DBB	PI(3)P per 50 µL
10 µM	-	10 µM	-	500 pmol
2.5 µM	4x	60 µL of 10 µM	180 µL	125 pmol
625 nM	4x	60 µL of 2.5 µM	180 µL	31.25 pmol
156 nM	4x	60 µL of 625 nM	180 µL	7.81 pmol
39.1 nM	4x	60 µL of 156 nM	180 µL	1.95 pmol
9.8 nM	4x	60 µL of 39.1 nM	180 µL	0.49 pmol

Table 2. Suggested Detection Plate Layout

	Standards and Controls			PI3K Reaction Samples (from step 3 of Kinase Reaction Section)								
Row	1	2	3	4	5	6	7	8	9	10	11	12
A	500 pmol PI(3)P			Reaction #1			Reaction #9			Reaction #17		
B	125 pmol PI(3)P			Reaction #2			Reaction #10			Reaction #18		
C	31.25 pmol PI(3)P			Reaction #3			Reaction #11			Reaction #19		
D	7.81 pmol PI(3)P			Reaction #4			Reaction #12			Reaction #20		
E	1.95 pmol PI(3)P			Reaction #5			Reaction #13			Reaction #21		
F	0.49 pmol PI(3)P			Reaction #6			Reaction #14			Reaction #22		
G	NL (No Lipid)			Reaction #7			Reaction #15			Reaction #23		
H	Blank			Reaction #8			Reaction #16			Reaction #24		

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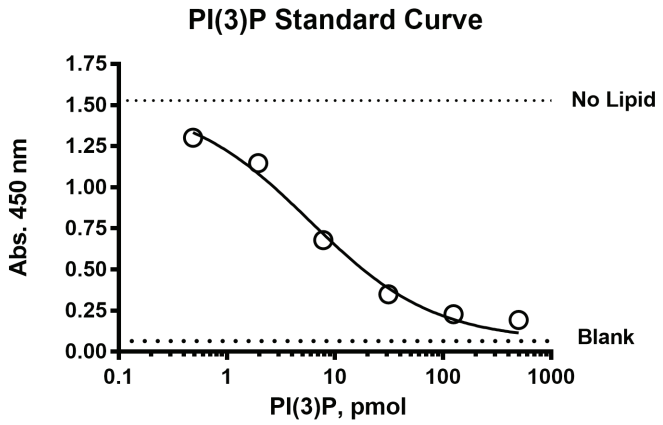
PI substrate* per assay point and PI(3)P concentration in the original 25 µL PI3-K reaction can be calculated as follows:



PI(3)P % conversion =
100 % x _____ pmol PI(3)P/500 pmol

PI(3)P concentration in quenched/diluted reaction mix =
_____ pmol PI(3)P/50 µL = _____ µM

PI(3)P conc. in original 25 µL reaction =
_____ pmol PI(3)P x (200 µL/50 µL)/25 µL = _____ µM

* If a different PI substrate concentration has been used in the PI3K reactions, please adjust the data analysis accordingly.

Reference (Background)

1. Engelman, J. A., Luo, J., and Cantley, L. C., (2006) The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism, Nat Rev Genet, 7, 606.

References (Publications)

1. Ramanan, R., et al., (2018) The Ancient Phosphatidylinositol 3-Kinase Signaling System Is a Master Regulator of Energy and Carbon Metabolism in Algae. Plant Physiology 177(3): p. 1050-1065.
2. Rajaram, M.V.S., et al., (2017) M. tuberculosis Initiated Human Mannose Receptor Signaling Regulates Macrophage Recognition and Vesicle Trafficking by FcRg-Chain, Grb2, and SHP-1. Cell Reports 21(1): p. 126-140.
3. Su, H., et al., (2017) VPS34 Acetylation Controls Its Lipid Kinase Activity and the Initiation of Canonical and Non-canonical Autophagy. Mol Cell.
4. Xu, D.-Q., et al., (2016) PAQR3 controls autophagy by integrating AMPK signaling to enhance ATG14L-associated PI3K activity. The EMBO Journal.

Related Products

Products	Catalog Number
Antibodies and Binding Proteins	
Anti-hVps34 for IP	Z-R015
Anti-hVps34 for Western	Z-R016
Anti-PI(3)P	Z-P003
PI(3)P Grip	G-0302
Substrates or Reaction Products	
PI	P-0004 P-0008, P-0008a P-0016
PI(3)P	P-3004 P-3008 P-3016
Labeled PI(3)P	C-03B6, C-03B6a, C-03F6, C-03M6, C-03M6a

Products	Catalog Number
Inhibitors	
Pan-PI3-K (Wortmannin)	B-0222
PI3-K Class 1 (LY294002, ZSTK474)	B-0294, B-0307
PI3-Kγ (1), PI3-Kγ (2),	B-0301, B-0302,
PI3-Kα (1), PI3-Kα (2), PI3-KΔ	B-0303, B-0304, B-0305, B-0021
Assays	
PIP3 Mass ELISA	K-2500s
PI(4,5)P2 Mass ELISA	K-4500
PI(3)P Mass ELISA	K-3300
PI3-K Activity ELISA: Pico	K-1000s
Enzymes	
Human p110α/p85α	E-2000
Human p110α (E545K)/p85α	P27-15H
Human p110β/p85α	P28-10H

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