PI3-Kinase Activity ELISA: Pico

K-1000s (96 tests)

Support: echelon@echelon-inc.com

Description: ELISA kit that measures class 1 PI3-kinase activity directly through its reaction product, PIP3. This assay can be used for in vitro PI3K activity in cancer tissue, cell lines, or compound screening for drug discovery.

Materials Provided

Catalog #	Description	Quantity
K-1001s	PI(3,4,5)P3 Detection Plate	1 plate
K-1008	PI(4,5)P2 Substrate, diC8	23 nmol
K-1003s	PI(3,4,5)P3 Standard, diC8	0.9 nmol
K-1006	PI(3,4,5)P3 Detector	20 µL
K-1005s	Detection Buffer	10 mL
K-SEC1	Secondary Detector	300 µL
K-TBST	10x TBS-T Buffer	20 mL
K-TMB1	TMB Solution	12 mL
K-STOPt	1 N H2SO4 Stop Solution	10 mL
K-KBZ	5x KBZ Kinase Reaction Buffer	4 mL
K-EDTA	100 mM EDTA Solution	500 µL
---	Incubation Plate	1 plate
---	Plate Sealers	3 seals

Quick Protocol

Isolate PI3K samples

↓

Perform PI3K reactions

↓

Prepare PIP3 Standards

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Incubate Standards and PI3K reactions with PIP3 Detector
1 hour at room temperature (Incubation Plate)

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Transfer samples from Incubation Plate to Detector Plate and incubate
1 hour at room temperature

↓

Remove samples and wash plate 3 times

↓

Add prepared Secondary Detector to plate and incubate
30 minutes at rooom temperature

↓

Remove Secondary Detector and wash plate 3 times

↓

Add TMP Substrate and incubate at room temparature for
30 minutes in the dark

↓

Add Stop Solution and read at 450 nm

Additional Materials Provided by User:

- Source of purified PI3-Kinase (human PI3-K p110α/p85α for positive control or inhibitor study, cat# E-2000)
- 450 nm absorbance plate reader
- 1 M DTT and 10 mM ATP stock solutions (store at -20°C in aliquots) for the Kinase Reaction Buffer.

Storage: Store kit at 4°C in a dark location. Store prepared reagents as indicated in the protocol.

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Background

The production of PI(3,4,5)P3 from PI(4,5)P2 by PI3-Kinases (PI3-K) is important in multiple cell signaling pathways. 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

The assay is a competitive ELISA in which the signal is inversely proportional to the amount of PI(3,4,5)P3 produced. After the PI3-Kinase reactions are complete, reaction products are first mixed and incubated with a PI(3,4,5)P3 detector protein, then added to the PI(3,4,5)P3-coated detection plate for competitive binding. A peroxidase-linked secondary detector and colorimetric detection is used to detect PI(3,4,5)P3 detector binding to the plate. The colorimetric signal is inversely proportional to the amount of PI(3,4,5)P3 produced by PI3-Kinase. The three major assay steps are outlined in the box below.

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Assay Notes

1. Use of the provided kinase reaction buffer has proven to improve PI3-Kinase activities. Optimization of DTT and ATP concentrations in the kinase reaction buffer may be desired. In our test, 2 mM DTT concentration in KBZ reaction buffer is optimal for p110alpha/p85a activity. As high as 150 µM ATP concentration in KBZ reaction buffer can be used to increase PI3-Kinase activity. For PI3-Kinase inhibitor study, lower ATP concentration to 25 µM or 50 µM may be desired.
2. Equilibrating PI(4,5)P2 substrate (K-1008) to room temperature before reconstitution is confirmed to improve PI3-K activity significantly. Optimization of the kinase reactions by PI(4,5)P2 substrate titration may be desired. Increasing PI(4,5)P2 substrate concentration will increase the PI3-Kinase activity but will also increase substrate competition with the PI(3,4,5)P3 detector. If a different PI(4,5)P2 substrate concentration is used, please adjust the PI(4,5)P2 concentration in the standard curve buffer accordingly. It is critical to keep PI(4,5)P2 concentrations in standard curve buffer the same as in the stopped PI3-K reactions.
3. The amount of enzyme to use per PI3-K reaction will vary according to your individual experiment and the cell type you are using. Whether you are using purified PI3-Kinase or enzyme immunoprecipitated from cell lysate, it's recommended to run an enzyme/cells titration to determine the optimum enzyme/cells amount to use for future assays. When using purified recombinant human PI3-K alpha from Echelon (Cat# E-2000), enzyme concentration of 0.05-0.25 µg/mL in reaction is suggested as a starting point. In testing, we found that enzyme immunoprecipitated from cell lysate containing 0.5 - 1 mg cellular protein (approximately 5 million cells), is usually sufficient for each PI3-K reaction (duplicate assay points).
4. Vigorous shaking will impair PI3-Kinase enzyme activity. If mixing is preferred during kinase reaction, gently flick the reaction tubes a few times every 30 minutes. If reactions are set up in a 96-well plate, it's not required to shake the reaction plate. However, shaking the reaction plate at or below 300 rpm on a plate shaker at 37°C slightly improves enzyme activity.
5. Beads used for immunoprecipitation need to be removed from kinase reactions before reactions are stored or detected. Do not freeze the beads at any time. See support protocol for immunoprecipitation of PI3K from cells.
6. The detection plate is composed of 12 of 8-well strips. Unused strip wells 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.
7. 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.
8. If tissue samples are used, flash freeze tissue in liquid nitrogen and ground to powder on dry ice before proceeding with the IP protocol below.

Protocol for the detection of PI3-K activity

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

Reagent Preparation

1. Bring plates (K-1001s, colored plate), lipids (K-1008, K-1003s), buffers (K-1005s, K-KBZ, K-TBST), EDTA solution (K-EDTA), TMB solution (K-TMB1) and Stop Solution (K-STOPt) to room temperature before use. Place detectors (K-1006, K-SEC1) on ice until use.
2. KBZ Reaction Buffer Prepare fresh KBZ reaction buffer from 5x KBZ Buffer, DTT and ATP stock solutions for use on the day of assay. Dilute 5x KBZ buffer (K-KBZ) 5-fold in dH2O and supplement with 2 mM DTT and 100 µM ATP (see Assay Note 1). Example for preparing 5 mL KBZ Reaction Buffer is as follows: 1 mL 5x KBZ Buffer + 10 µL 1 M DTT + 50 µL 10 mM ATP + 3,940 µL dH_2O
3. PI(4,5)P2 Substrate Prepare a 100 µM PI(4,5)P2 substrate stock solution by adding 230 µL dH2O to the vial of diC8 PI(4,5)P2 substrate (K-1008). Vortex the vial shortly to fully reconstitute the lipid. Spin down and place at room temperature until use. Further dilute the required amount of PI(4,5)P2 substrate in KBZ Reaction Buffer for a 10 µM (2x concentration) working solution (see Assay Note 2). Place substrate working solution at room temperature until use. The 100 µM PI(4,5)P2 stock solution will be used again to prepare Standard Curve Buffer in step 5.
   A. Store remaining 100 µM PI(4,5)P2 substrate at -20ºC for up to 3 months. A 500 µL working solution can setup 15 PI3-K reactions (30 assay points).
   B. 500 µL of 10 µM PI(4,5)P2 substrate = 50 µL of 100 µM PI(4,5)P2 stock + 450 µL of KBZ Reaction Buffer
4. Kinase Stop Solution Prepare a Kinase Stop Solution by supplementing KBZ Reaction Buffer with 4 mM EDTA. 1.5 mL Kinase Stop Solution can stop 15 PI3-K reactions (30 assay points). Place Kinase Stop Solution at room temperature until use.
   A. 1.5 mL of Kinase Stop Solution = 1.44 mL of KBZ Reaction Buffer + 60 µL of 100 mM EDTA (K-EDTA)
5. Standard Curve Buffer Standard Curve Buffer will be used to dilute the PI(3,4,5)P3 standards. On the day of ELISA assay, prepare the Standard Curve Buffer by supplementing KBZ

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Reaction Buffer with 2 µM PI(4,5)P2 and 2.4 mM EDTA. 1.6 mL of Standard Curve Buffer will be sufficient for triplicate standards and controls. Place Standard Curve Buffer at room temperature until use.

6. 1.6 mL Standard Curve Buffer = 1,530 µL KBZ Reaction Buffer + 32 µL 100 µM PI(4,5)P2 + 38.4 µL 100 mM EDTA

7. PI(3,4,5)P3 Standard On the day of the first ELISA assay, prepare a 3.6 µM PI(3,4,5)P3 standard stock solution by adding 250 µL dH2O to the vial of diC8 PI(3,4,5)P3 standard (K-1003s). Vortex the vial shortly to fully reconstitute the lipid. Spin down and place at room temperature before use. Unused portion of 3.6 µM PI(3,4,5)P3 standard stock can be stored at -20ºC for up to 3 months.
8. TBS-T Buffer Prepare TBS-T buffer by diluting 20 mL of the provided 10x TBS-T buffer (K-TBST) with 180 mL dH2O.

Kinase reaction

1. Isolate or prepare PI3-Kinase enzyme according to usual protocols. See attached support protocol for immunoprecipitation of Class IA PI3-K enzymes from cells. Prior to use, dilute the needed amount of PI3-Kinase to a 2x concentration in the KBZ Reaction Buffer (see Assay Note 3).
2. PI3-Kinase reactions can be set up in micro centrifuge tubes.
   A. For each 60 µL PI3-K reaction (for duplicate assay points): combine 30 µL of 10 µM PI(4,5)P2 substrate (300 pmol) and 30 µL of PI3-Kinase (2x concentration). You may also include an Enzyme Only control by replacing 30 µL of substrate with 30 µL of KBZ Reaction Buffer.
   B. Seal the kinase reactions and let them procee d for a certain time, usually 2-3 hours at 37°C (see Assay Note 4).
   C. Stop each 60 µL kinase reaction by adding 90 µL of Kinase Stop Solution.
   D. If PI3-K enzymes are bound to beads in kinase reactions, centrifuge to separate the beads before transferring the reaction supernatant to the Incubation Plate (colored plate) to proceed with Incubation and Detection (see Assay Note 5). Option: Transfer reaction supernatant to clean tubes and store at -20°C for up to 2 weeks. Do not freeze the beads. ELISA can be run on another day.

Incubation and Detection (ELISA)

We suggest that standards and controls be run in duplicate or triplicate. The Incubation/Detection Plate layout below shows an example plate layout with duplicate standards and controls.

1. Prepare PI(3,4,5)P3 standards and controls on day of the ELISA assay.
   A. From the 3.6 µM PI(3,4,5)P3 stock prepared earlier, prepare a 1.08 µM PI(3,4,5)P3 standard by adding 90 µL of the 3.6 µM stock solution to 210 µL of the Standard Curve Buffer.
   B. Make five 3-fold serial dilutions from the 1.08 µM PI(3,4,5)P3 with Standard Curve Buffer, as outlined in Table 1, below.
   C. 100 µL of previous dilution + 200 µL Standard Curve Buffer = 300 µL of next dilution.
   D. Pipet 60 µL/well of each standard solution in triplicate or duplicate to rows B through G of the Incubation Plate (colored). See Table 2 for suggested layout.
   E. Pipet 60 µL/well of the Standard Curve Buffer to the No Enzyme control wells in row H of the Incubation Plate.
   F. Pipet 60 µL/well of KBZ Reaction Buffer to the Blank control wells in row A of the Incubation Plate.
   G. Optional: Pipet 60 µL/well of KBZ Reaction Buffer to selected No Lipid control wells of the Incubation Plate.
   H. Pipet 60 µL/well of Detection Buffer (K-1005s) to the Blank control wells in row A.
2. Transfer 60 µL/well of each stopped kinase reaction into 2 wells of the Incubation Plate for duplicate data points.
3. Incubate with PI(3,4,5)P3 detector.
   A. Briefly centrifuge (do not vortex) the vial of PI(3,4,5)P3 detector (K-1006). Dilute the PI(3,4,5)P3 detector 1:800 in Detection Buffer (K-1005s). Make only enough working solution of detector for the current assay and store the remainder of the PI(3,4,5)P3 detector at 4°C for future use.

Table 1

PIP3 Standards and Controls	PIP3 per 50 µL solution
1.08 µM	54 pmol
360 nM	18 pmol
120 nM	6 pmol
40 nM	2 pmol
13.3 nM	0.67 pmol
4.4 nM	0.22 pmol
No Enzyme control	0 pmol (with substrate)
No Lipid control (optional)	0 pmol

Table 2, Suggested Incubation Plate Layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Sample #1	Sample #1	Sample #9	Sample #9	Sample #17	Sample #17	Sample #25	Sample #25	Sample #33	Sample #33
B	54 pmol PIP3	54 pmol PIP3	Sample #2	Sample #2	Sample #10	Sample #10	Sample #18	Sample #18	Sample #26	Sample #26	Sample #34	Sample #34
C	18 pmol PIP3	18 pmol PIP3	Sample #3	Sample #3	Sample #11	Sample #11	Sample #19	Sample #19	Sample #27	Sample #27	Sample #35	Sample #35
D	6 pmol PIP3	6 pmol PIP3	Sample #4	Sample #4	Sample #12	Sample #12	Sample #20	Sample #20	Sample #28	Sample #28	Sample #36	Sample #36
E	2 pmol PIP3	2 pmol PIP3	Sample #5	Sample #5	Sample #13	Sample #13	Sample #21	Sample #21	Sample #29	Sample #29	Sample #37	Sample #37
F	0.67 pmol PIP3	0.67 pmol PIP3	Sample #6	Sample #6	Sample #14	Sample #14	Sample #22	Sample #22	Sample #30	Sample #30	Sample #38	Sample #38
G	0.22 µM PIP3	0.22 µM PIP3	Sample #7	Sample #7	Sample #15	Sample #15	Sample #23	Sample #23	Sample #31	Sample #31	Sample #39	Sample #39
H	No Enzyme	No Enzyme	Sample #8	Sample #8	Sample #16	Sample #16	Sample #24	Sample #24	Sample #32	Sample #32	Sample #40	Sample #40

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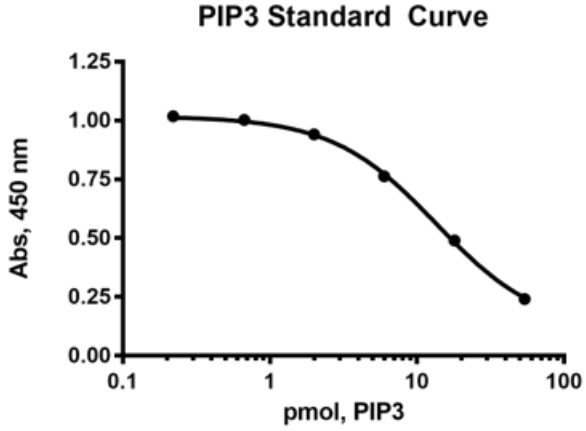
B. Add 60 µL /well of above diluted PI(3,4,5)P3 detector to all control, standard, and stopped reaction wells of the Incubation Plate except the blank controls in row A.
C. Seal the Incubation Plate and incubate for 60 minutes at room temperature with gentle agitation on a plate shaker.

4. After incubation, transfer 100 µL of standards, controls, and reactions from the Incubation Plate to corresponding wells of the Detection Plate (K-1001s). Seal plate and incubate for 60 min at room temperature with gentle agitation on a plate shaker (see Assay Notes 6 and 7).
5. Discard solutions from the detection plate and wash the wells 3 times with 200 µL/well of TBS-T.
6. Briefly centrifuge the vial of secondary detector (K-SEC1). Dilute the secondary detector 1:80 with TBS-T and mix by gently invert the tube 10 times. Dilute ONLY the amount you will use for the current assay and store the remainder of the secondary detector at 4°C for future use. Discard the last TBS-T wash from plate, and add 100 µL of diluted secondary detector to each well of the detection plate. Seal the plate and incubate for another 30 minutes at room temperature on a plate shaker.
7. Discard solutions from the detection plate and wash the wells 3 times with 200 µL/well of TBS-T.
8. Discard the last TBS-T wash from plate completely and immediately add 100 µL of room temperature TMB solution (K-TMB1) to each well. Allow color to develop for 30 minutes in dark.
9. Add 50 µL of 1 N H_2SO_4 stop solution (K-STOPt) to each well to stop color development. Blue color will change to yellow color upon addition of stop solution. Eliminate any big air bubbles present in wells before reading the plate. Caution: Use caution when dealing with corrosive 1 N H2SO4 stop solution.
10. Read absorbance at 450 nm on a plate reader.

Quantification of Samples:



PI3-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(3,4,5)P3 in pmol per standard to generate a standard curve using sigmoidal dose-response (variable slope) correlation. For best results, constrain standard curve top and bottom with No Enzyme control and Blank control OD values, respectively. Determine the PI(3,4,5)P3 level in pmol by interpolation from absorbance values obtained from the enzyme reactions. PI3-K activity in your samples can be estimated by the percentage conversion from initial 100 pmol of PI(4,5)P2 per assay point. There are 100 pmol of combined lipids, PI(3,4,5)P3 product plus remaining PI(4,5)P2 substrate, in each assay point.

Support Protocols

Immunoprecipitation of Class IA PI3-Kinase from cells

1. Grow cells to 80% confluence in 10 cm dishes.
2. Induce quiescence by incubating overnight in serum-free medium containing 0.5% insulin-free BSA.
3. Stimulate cells with 100 nM insulin in serum-free medium for 10 minutes at 37°C. Optional: stimulate cells with 20 – 40 ng/mL PDGF in serum-free medium for 3-5 min at room temperature.
4. Remove solution and place cells on ice. Add 10 mL per dish of ice-cold Buffer A (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 1 mM CaCl2, 1 mM $MgCl_2$, and 1 mM sodium orthovanadate). Rinse three times with this solution.
5. Remove Buffer A and add 1 mL of ice cold Lysis Buffer (Buffer A plus 1% NP-40 and 1 mM PMSF). Keep plates on ice for 20 minutes.
6. Scrape cells from dish, transfer to 1.5 mL microcentrifuge tubes. Centrifuge for 10 minutes to sediment insoluble material.
7. Transfer supernatant to new tubes, add 5 µL of anti-PI3-Kinase antibody (Millipore, catalog # 06-195) to each tube. Incubate for one hour at 4°C with gentle rotation.
8. Add 60 µL of 50% slurry of Protein A-agarose beads in PBS to each tube. Incubate with gentle rotation at 4°C for one to two hours (incubate over night if desired).
9. Collect immunoprecipitated enzyme by centrifuging 5 seconds, and wash with freshly prepared buffers as follows:
   A. Three times with Buffer A plus 1% NP-40
   B. Three times with 0.1 M Tris-HCl, pH 7.4; 5 mM LiCl, and 1 mM sodium orthovanadate.
   C. Twice with TNE (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) containing 1 mM sodium orthovanadate.
   D. Twice with KBZ Reaction Buffer (See Reagent Preparation 2 on Page 2 for buffer recipe and preparation).
10. Aspirate last wash completely, add 30 µL of KBZ Reaction Buffer to cover the beads. Proceed immediately to PI3-K reactions by adding 30 µL of 10 µM PI(4,5)P2 substrate as described in the Basic Assay Protocol.

Detection of total PI3-Kinase activity from cell lysates

Total PI3-K activity from cell lysates can be detected and quantified using the K-1000s kit. For relative quantification of total PI3-K activity, all samples should be normalized to total protein.

Please see Ding et al. 2020 under 'Protocol References' for additional details on sample preparation and detection.

General protocol:

1. Lyse cells in ice-cold lysis buffer, incubate samples on ice for 10-20 min, centrifuge at 10,000g for 10 min at 4°C, measure protein concentration, take 20 – 30 µg protein per sample out for each PI3K reaction.
2. Add 20 – 30 µg (≤ 6 µL) cell lysate to eppendorf tube and mix with equal volume of 2x KBZ. Adjust total volume to 30 µL with 1x KBZ
3. Set up PI3K reaction: add 30 µL of 10 µM PI(4,5)P2 substrate in 1xKBZ to each 30 µL of sample. Let the reaction proceed without shaking for 2-3 hours at 37°C. Experiments should be ran to determine the optimal reaction time for your samples.
4. Prepare PI(3,4,5)P3 standards with this standard curve buffer: 1x KBZ buffer containing 2 µM PI(4,5)P2, 2.4 mM EDTA and ≤ 4% of

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lysis buffer strength as in the stopped PI3K reactions in step 4. Lysis buffers may affect assay performance and should be tested with the PIP3 standards and controls before running samples.

5. Stop kinase reactions by adding 90 µL of Kinase Stop Solution (4 mM EDTA in 1x KBZ) to each 60 µL PI3K reaction.
6. Follow Incubation and Detection (ELISA) procedure of the current K-1000s Basic Assay protocol to measure PI(3,4,5)P3 amount in samples.

Buffer Recipes:

Lysis buffer:

Lysis buffer 1: 20 mM Tris-HCl, pH 7.4, 137 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, and 1% NP-40. Add sodium orthovanadate to 1 mM freshly before use.

Lysis Buffer 2: RIPA lysis buffer supplemented with 1 mM sodium orthovanadate.

Lysis Buffer 3: 50 mM Tris pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.5% Triton, 1.5 mM Na3VO4, 50 mM NaF, 10mM sodium pyrophosphate.

Note: it is suggested, but optional, to add a protease inhibitor cocktail without EDTA prior to use.

1x KBZ: dilute 5x KBZ, provided with the kit and add 100 µM ATP and 2 mM DTT freshly from 10 mM ATP and 1M DTT stocks.

2 mM DTT is optimal for p110a/p85a activity.

Note: Equilibrate PIP2 substrate (K-1008) and PIP3 standard (K-1003s) to room temperature (≥ 15 min) before reconstitution in DI water. Vortex to mix and spin down. Leave vials at room temperature until use. Store at -20°C after use.

Assay References:

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Protocol References:

Ding Q, Sethna F, Wu X-T, Miao Z, Chen P, Zhang Y, et al. Transcriptome signature analysis repurposes trifluoperazine for the treatment of fragile X syndrome in mouse model. Communications Biology. 2020;3(1):127.

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Related Products

Products	Catalog Number
Enzymes	
Human PI3K p110α/p85α	E-2000
Human PI3K p110α	P27-15H
Mouse PI3K p110α/p85α	P27-18H
Human PI3K p110β/p85α	P28-10H
PTEN Enzyme	E-3000
SHIP2 Enzyme	E-1000
Substrates	
PI(4,5)P2	P-4504, P-4508, P4516
Labeled PI(4,5)P2	C-45B6, C-45B6a, C-45F6, C-45M6
Reaction Buffers	
PI3K Reaction Buffer	K-KBZ
SHIP2 Reaction Buffer	K-S2RB

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
SHIP1	B-0341, B-0342, B-0343
PTEN Inhibitor	B-0350, B-0351
Assays	
PIP3 Mass ELISA	K-2500s
PI(4,5)P2 Mass ELISA	K-4500
PI(3)P Mass ELISA	K-3300
Class III PI3-K ELISA	K-3000
Screening Services	
PI3-K alpha Inhibitor Screen	T-1300α
PI3-K beta Inhibitor Screen	T-1300β

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