Echelon Biosciences Inc.

PI3-Kinase Fluorescence Polarization Activity Assay

K-1100S (384 tests)

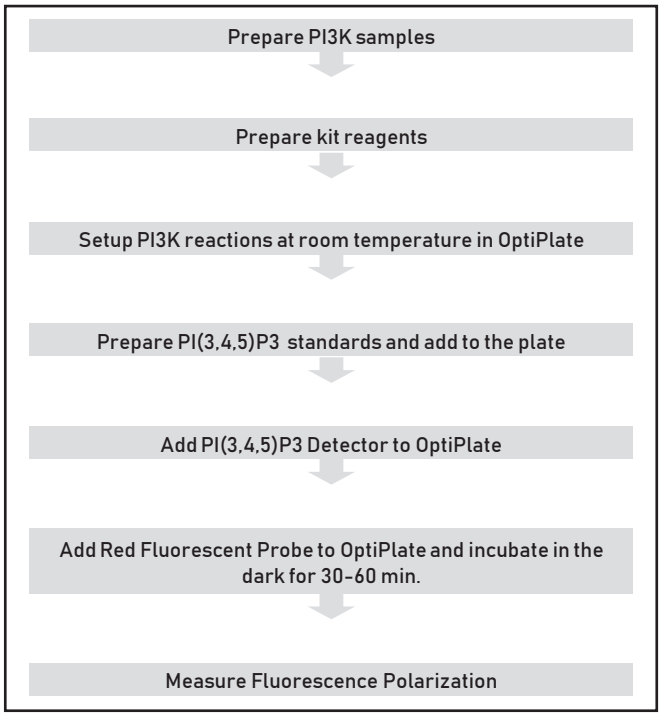
Support: echelon@echelon-inc.com

Description: This assay determines in vitro PI3-K activity through its end product, PIP3.

Materials Provided

Catalog #	Description	Amount
K-1101L	PI(4,5)P2 Substrate	69 µg
K-1003s	PI(3,4,5)P3 Standard	0.88 µg
K-1103	PI(3,4,5)P3 Detector	1 pellet
K-1104	2.5 uM Red Fluorescent Probe	50 µL
---	OptiPlate-384 F	1 plate
K-KBZ	5X KBZ buffer	4 mL
K-ATP1	10 mM ATP	50 µL
K-DTT1	DTT	50 µmol
---	Acetate plate seal	1 seal

Quick Protocol



Additional Materials Provided by User:

- Source of PI 3-Kinase. Enzyme may be immunoprecipitated or purified (PI3Kα Echelon cat# E-2000). Use of crude lysates is not recommended.
- Fluorescence plate reader equipped for Fluorescence Polarization using red fluorophores with appropriate filters (550 nm excitation/580 nm polarizing emission filters)

Storage: Upon receipt, store the kit at –20°C. Some components are light sensitive. Under proper storage conditions, this product is stable for at least 6 months from date of receipt. Opened and reconstituted solutions are less stable. All components and solutions should be protected from excessive light and heat.

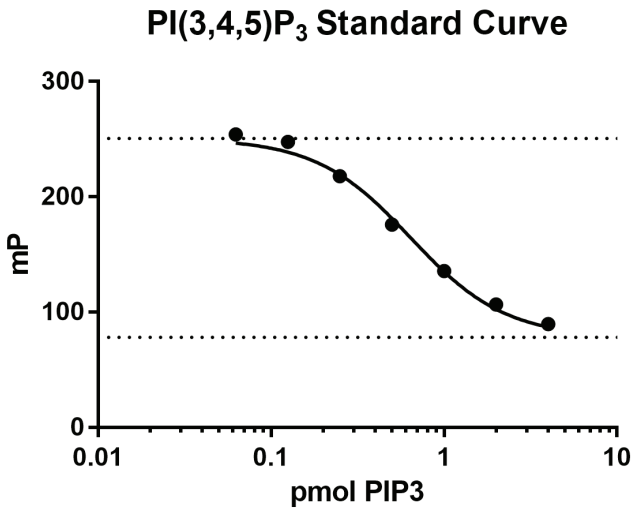
Echelon Biosciences Inc.
Ph: 801-588-0455
Fax: 801-588-0497
Echelon-inc.com

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, extraction of radioactive products, and separation using thin-layer chromatography. The fluorescence polarization PI3-K activity assay developed by Echelon Biosciences, Inc. allows the user to determine PI3-K activity using a homogenous mix and read format, eliminating the need for radioactivity, organic solvents, and thin layer chromatography. The assay can be adapted for HTS applications.

Assay Design

The assay is a competitive assay in which the degree of polarization (mP) of the fluorescent PI(3,4,5)P3 probe is inversely proportional to the amount of PI(3,4,5)P3 produced by PI3-K activity. After the PI3-K reactions are complete, reaction products are mixed with a PI(3,4,5)P3 detector protein and the fluorescent PI(3,4,5)P3 probe. Polarization (mP) values decrease as probe binding to the PI(3,4,5)P3 detector is displaced by PI(3,4,5)P3 produced by enzymatic activity and the amount of unbound fluorescent probe in the mixture increases. The graph (below) shows a PI(3,4,5)P3 standard curve using sigmoidal dose-response (variable slope) curve fit. The Top and Bottom of the standard curve have been constrained to Enzyme Only control and Probe Alone control signals, respectively.



Assay Notes

1. We suggest running duplicate or triplicate assay points for each enzyme reaction.
2. The 5x KBZ buffer has been tested with PI3-K isoforms (α, β, γ) and has shown to improve PI3-K activity. If you have your own PI3-K reaction buffer it can be substituted in the assay where the protocol says "Reaction Buffer".
3. The amount of enzyme to use per 100 pmol of substrate will vary according to your individual experiment. Whether you are using purified PI3-K or enzyme immunoprecipitated from cell extracts, you will need to try reactions using different amounts of enzyme to determine the optimum amount. In our testing, we found 10 ng of purified recombinant PI3-Kα (E-2000) per point, or enzyme immunoprecipitated from cell lysates containing 5 ug cellular protein (approximately 50,000 cells), to be sufficient. A support protocol for immunoprecipitation of PI3-K from cell lysates is provided below.
4. The assay was developed using a Fusion Alpha Universal Microplate reader equipped for Fluorescence Polarization. The sensitivity of the assay and the amount of substrate, detector, and fluorescent probe required for each assay point may vary depending on the specific fluorescence polarization detection system you are using.
5. The provided OptiPlate-384 F assay plate works well with PI3K reactions. If you choose to use a different plate, please test PI3K reactions in the assay to make sure the well surface will not inhibit PI3-K reactions. Corning plates with NBS surface are not recommended.
6. The concentrations of DTT, ATP, and PI(4,5)P2 substrate suggested are based on our experience using recombinant PI3Kα. The assay conditions used in your enzyme reaction can affect your enzyme activity and the activity of a potential inhibitor. You may want to titrate DTT, ATP, and/or PI(4,5)P2 substrate to determine the optimum conditions for your experiments.
7. Suggested controls include:
   - Buffer Only: 5 µL of 2X Reaction Buffer, 5 µL of enzyme diluent, 15 µL of Detector Diluent. This control may be required as FP Blank for plate reading in FP mode.
   - Probe Alone: 5 µL of 2X Reaction Buffer, 5 µL of enzyme diluent, 10 µL of Detector Diluent, and 5 µL of 50 nM Probe.
   - No enzyme: 5 µL of 2X Reaction Buffer, 5 µL of enzyme diluent, 10 µL of PI(3,4,5)P3 Detector, and 5 µL of 50 nM Probe.

Reagent Preparation

Place PI(3,4,5)P3 Detector (K-1103), Red Fluorescent Probe (K-1104), ATP (K-ATP1), and DTT (K-DTT1) on ice. Equilibrate lipids (K-1101L and K-1003s) to room temperature before use. Leave buffers (K-KBZ, K-1105) and assay plate at room temperature. Unless indicated otherwise, all steps are performed at room temperature. Centrifuge each vial briefly prior to open its cap.

PI(3,4,5)P3 Standard (K-1003s) Add 22.4 uL of dH2O to the vial of PI(3,4,5)P3 for a 40 µM solution; vortex to fully reconstitute; spin down and leave vial at room temperature prior to use. Enough standard is provided for approximately 10 separate dilution series. Store frozen at –20°C after use for up to 6 months. Multiple freeze-thaw cycles do not affect stability.

DTT (K-DTT1) Add 50 µL of dH2O to the vial of DTT for a 1 M solution and vortex to fully reconstitute; spin down and keep on ice. Once reconstituted DTT is less stable, store frozen at –20°C after use for up to 1 month.

ATP (K-ATP1) One vial with 50 µL of 10 mM ATT. Store frozen at –20°C after use.

PI(4,5)P2 Substrate (K-1101L) Add 100 µL of dH2O to the vial of PI(4,5)P2 for a 800 µM solution; vortex to fully reconstitute; spin down and leave vial at room temperature prior to use. There is substrate for up to 800 assay points using 100 pmol per assay point (Final concentration in reaction: 10 µM). Store frozen at –20°C after use for up to 6 months.

2X Reaction Buffer Dilute 5x KBZ buffer (K-KBZ) 2.5-fold in dH2O and supplement with 4 mM DTT, 20 µM PI(4,5)P2 substrate, and 50 µM ATP. Only prepare the amount needed. Make fresh before each use. Store remaining 5x KBZ at 4°C.

Echelon Biosciences Inc.
Ph: 801-588-0455
Fax: 801-588-0497
Echelon-inc.com

For 2 mL of 2X Reaction Buffer add:
800 µL 5x KBZ Buffer
8 µL 1M DTT
10 µL 10 mM ATP
1,132 µL dH2O
50 µL 800 µM PI(4,5)P2 solution

1X Reaction Buffer Prepare a 1X reaction buffer for preparation of the PI(3,4,5)P3 standards by adding 250 µL of the 2X Reaction Buffer to 250 µL of dH2O or your enzyme diluent.

PI(3,4,5)P3 Detector (K-1103) Each pellet contains enough PI(3,4,5)P3 detector for 400 assay points. Reconstitute in 4.25 mL of Detector Diluent (K-1105) for a 250 nM stock. Do not vortex. Mix gently by pipetting and keep solution on ice. Aliquot and store frozen at –80°C. Although the reagent is stable for several weeks through multiple freeze-thaw cycles, we recommend aliquoting to minimize this variable.

Red Fluorescent Probe (K-1104) Prior to use, dilute 2.5 µM Red Fluorescent Probe (K-1104) 50-fold in Detector Diluent (K-1105) for a 50 nM working solution. Store remaining Red Fluorescent Probe frozen at –20°C. Multiple freeze-thaw cycles do not affect stability. **IMPORTANT: Minimize exposure of this reagent to light.**

PI3-Kinase Reaction
Conditions for enzyme activity will depend on the characteristics and source of the enzyme used in each specific application. The following protocol has been used at Echelon to detect the activity of a recombinant 6xHis-tagged PI3-Kα and is given as a guideline. Reaction Buffers and working solutions of Substrate, Enzyme, and Inhibitors should be freshly made prior to each experiment. Substrate concentration in the 1X reaction buffer is 10 µM.

Set up PI3-kinase reactions in 10 µL volume per assay point. For best result, set up reactions directly in the wells of the provided black OptiPlate-384 F.
For each 10 µL reaction add the following:

- 5 µL PI3-kinase enzyme. If inhibitors are to be added to the reaction they should be added with the enzyme and a 5 min pre-incubation of the enzyme with the inhibitor is suggested.
  Note: We suggest a "no enzyme" control.
- 5 µL 2X Reaction Buffer.

Incubate at 25 to 37°C for appropriate time period, depending on the activity of your enzyme. The exact amount of enzyme and conditions of incubation will vary with different enzyme preparations and will need to be optimized for each specific application.

In our hands, each assay point uses approximately 10 ng of 6xHis-tagged recombinant PI3-Kα (E-2000) per 100 pmol of PI(4,5)P2 substrate, with 1 hour incubation at 37°C.

Protocol for Fluorescence Polarization Assay
Please read this entire section and the Assay Notes section before beginning the assay.

1. Preparation of PI(3,4,5)P3 Standard Along with the enzyme reactions, a standard curve of PI(3,4,5)P3 is created to allow determination of the amount of PI(3,4,5)P3 produced. From a 4 µM stock of PI(3,4,5)P3, make six 2-fold serial dilutions in 1X Reaction Buffer, as outlined in the table below. Use 10 µL of each dilution to set up assay points containing 0.625, 1.25, 2.5, 5, 10, 20, and 40 pmol PI(3,4,5)P3. Also set up a standard containing 10 µL 1X Reaction Buffer alone with no PI(3,4,5)P3 competitor. It is suggested that the standards are run in duplicate or triplicate. Prepare only what is needed for each individual experiment.

2. Quenching and Detection of PI 3-Kinase Activity Mix in the following order for a total of 25 µL per well in the 384-well plate provided with the kit.
   a. 10 µL of enzyme reactions, controls, or PI(3,4,5)P3 standards in each well.
      Note: we suggest running a Probe Alone and Buffer Only control with each experiment. See assay notes for more information.
   b. 10 µL of 250 nM stock of PI(3,4,5)P3 Detector.
      Note: Addition of this reagent quenches the kinase reaction. If you are running a time course of enzyme activity, the PI(3,4,5)P3 Detector solution can be added to quench at various time points and the Red Fluorescent Probe solution can be added after all reactions are complete.
   c. 5 µL of 50 nM Red Fluorescent Probe working solution.

3. Incubation and Measurement Tap plate to mix gently. Seal plate and protect from exposure to light. Incubate in a dark location for 30 - 60 minutes to equilibrate. Incubations may be as long as six hours with minimal effect on final measurements. Measure fluorescence polarization using an appropriate instrument and filter set compatible with TAMRA dyes. (550 nm excitation/580 nm polarizing emission filters will give satisfactory results.). Values obtained for enzyme reactions can be compared to the standard curve to determine conversion of substrate to PI(3,4,5)P3.

Table 1. PIP3 Standards

Concentration of PIP_3	PIP_3/10µL	100 µM stock or previous dilution	1X Reaction Buffer
4.0 µM	40 pmol	8 µL (40 µM PI(3,4,5)P_3 Stock Soln.)	72 µL
2.0µM	20 pmol	40 µL (4 µM Solution)	40 µL
1.0 µM	10 pmol	40 µL (2 µM Solution)	40 µL
0.5 µM	5 pmol	40 µL (1 µM Solution)	40 µL
0.25 µM	2.5 pmol	40 µL (0.5 µM Solution)	40 µL
0.125 µM	1.25 pmol	40 µL (0.25 µM Solution)	40 µL
0.0625 µM	0.625 pmol	40 µL (0.125 µM Solution)	40 µL
0 µM	0 pmol	-	40 µL

Echelon Biosciences Inc.
Ph: 801-588-0455
Fax: 801-588-0497
Echelon-inc.com

Support Protocols

Immunoprecipitation of PI3-Kinase

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. Remove medium, and stimulate cells with 100 nM insulin for 10 minutes at 37°C.
4. Remove solution and place cells on ice. Add 10 mL per dish of ice-cold Buffer A (137 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM CaCl2, 1 mM MgCl2, and 0.1 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 microfuge tubes. Centrifuge for 10 minutes at high speed to sediment insoluble material.
7. Transfer supernatent 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 mL of a 50% slurry of Protein A-agarose beads in PBS to each tube. Incubate with mixing for one hour at 4°C.
9. Collect immunoprecipitated enzyme by centrifuging 5 seconds, and wash with freshly prepared buffers as follows:

   Three times with Buffer A plus 1% NP-40
   Three times with 0.1 M Tris-HCl, pH 7.4; 5 mM LiCl, and 0.1 mM sodium orthovanadate.
   Twice with TNE (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA) containing 0.1 mM sodium orthovanadate.
10. Remove last wash as completely as possible. Wash twice with 1X KBZ and proceed immediately with kinase reactions as described in the Basic Protocol.

References

1. Miller MS, Maheshwari S, McRobb FM, Kinzler KW, Mario Amzel L, Vogelstein B, et al. Identification of allosteric binding sites for PI3KÎ± oncogenic mutant specific inhibitor design. Bioorganic & Medicinal Chemistry. 2017.
2. Heffron TP, Heald RA, Ndubaku CO, Wei B, Augustin M, Do S, et al. The Rational Design of Selective Benzoxazepin Inhibitors of the α-Isoform of Phosphoinositide 3-Kinase Culminating in the Identification of (S)-2-((2-(1-isopropyl-1H-1,2,4-triazol-5-yl)-5,6-dihydrobezo[f]imidazo [1,2-d][1,4]oxazepin-9-yl)oxy)propanamide (GDC-0326). Journal of medicinal chemistry. 2016.
3. Safina BS, Baker S, Baumgardner M, Blaney PM, Chan BK, Chen Y-H, et al. Discovery of Novel PI3-Kinase d Specific Inhibitors for the Treatment of Rheumatoid Arthritis: Taming CYP3A4 Time-Dependent Inhibition. Journal of medicinal chemistry. 2012;55(12):5887–900.
4. Dehnhardt CM, Mansour Tarek S. Lead Optimization of N-3-Substituted 7-Morpholinotriazolopyrimidines as Dual Phosphoinositide 3-Kinase/Mammalian Target of Rapamycin Inhibitors: Discovery of PKI-402. J Med Chem. 2009.
5. Ma H, Deacon S, Horiuchi K. The Challenge of Selecting Protein Kinase Assays for Lead Discovery Optimization. Expert Opinion on Drug Discovery. 2008;3(6):607-21.
6. Carson JD, Van Aller G, Lehr R, Sinnamon RH, Kirkpatrick RB, Auger KR, et al. Effects of oncogenic p110alpha subunit mutations on the lipid kinase activity of phosphoinositide 3-kinase. Biochem J. 2008;409(2):519-24..
7. Nagoshi T, Matsui T, Aoyama T, Leri A, Anversa P, Li L, et al. PI3K rescues the detrimental effects of chronic Akt activation in the heart during ischemia/reperfusion injury. J Clin Invest. 2005;115(8):2128-38.
8. Drees BE, Weipert A, Hudson H, Ferguson CG, Chakravarty L, Prestwich GD. Competitive fluorescence polarization assays for the detection of phosphoinositide kinase and phosphatase activity. Comb Chem High Throughput Screen. 2003;6(4):321-30.

Related Products

Products	Catalog Number
Assays and Enzymes	
PI3-Kinase alpha, active (PI3Ka)	E-2000
PI3-Kinase Activity ELISA: Pico	K-1000s
Class III PI3K Elisa Kit	K-3000
Antibodies	
Anti-PtdIns(3,4,5)P3 IgG	Z-P345b
Biotinylated Anti-PtdIns(3,4,5)P3 IgM	Z-B345

Products	Catalog Number
Lipids	
PIP3	P-3908, P-3916
BODIPY® TMR- PtdIns(3,4,5)P3	C-39M6a
PI(3,4,5)P3 Grip (Grp1-PH)	G-3901
Inhibitors	
Wortmannin	B-0222
LY294002	B-0294

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

Echelon Biosciences Inc.
Ph: 801-588-0455
Fax: 801-588-0497
Echelon-inc.com