Echelon Biosciences Inc.

Luminescent PI3-Kinase Activity Assay

K-1300 (500 tests)

Support: echelon@echelon-inc.com

Materials Provided

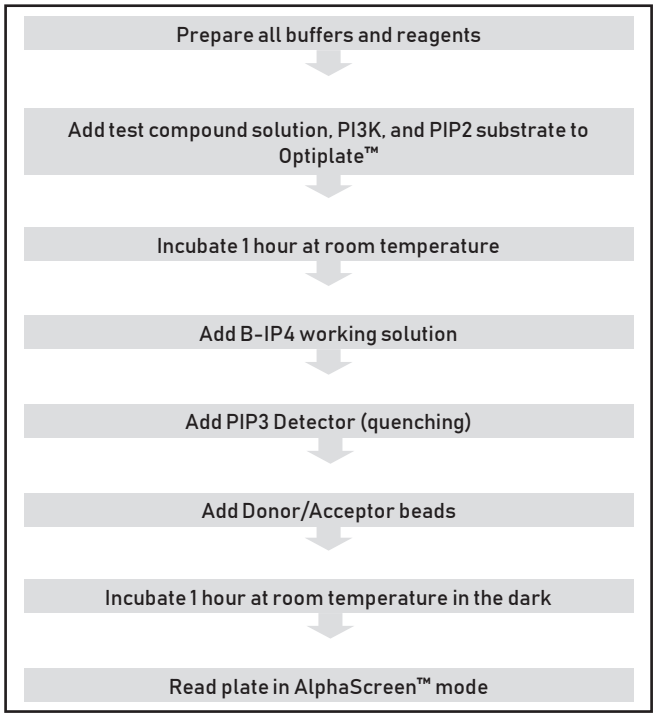
Catalog #	Description	Amount
K-1301L	PI(4,5)P2 Substrate	100 nmol
K-1302L	PI(3,4,5)P3 Standard	0.5 nmol
K-1303	PI(3,4,5)P3 Detector	5 ug
K-1305L	Biotinylated I(1,3,4,5)P4	0.15 nmol

Additional Materials Provided by User:

- PI3-K Reaction Buffer (cat# K-KBZ, optional)
- Detection Buffer
- PI3-Kinase Enzyme (cat# E-2000, optional)
- AlphaScreen™ GST Detection Kit (PerkinElmer cat# 6760603C, M, or R)
- 384-well white Optiplate™ (PerkinElmer cat# 6007299)
- Plate reader equipped for AlphaScreen™ Detection

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.

Quick Protocol



This product is intended for use with the Amplified Luminescent Proximity Homogenous Assay (AlphaScreen™) technology and requires the researcher to obtain donor and acceptor beads from the AlphaScreen™ GST (Glutathione-S-Transferase) Detection Kit, which must be purchased separately from PerkinElmer Life Sciences. A microplate reader with AlphaScreen™ capabilities is also required.

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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, 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

AlphaScreen™ technology is based on the emission of light (520-620nm) by Acceptor beads activated by the proximity of Donor beads. The interaction of biotinylated-I(1,3,4,5)P4 and the PI(3,4,5)P3 detector protein brings both Acceptor and Donor beads together, producing a cascade of chemical reactions and leading to the amplified AlphaScreenTM signal. This highly amplified signal is detected upon excitation of the Donor beads at 680 nm when singlet state oxygen (102) molecules are generated and diffuse to excite Acceptor beads.

When PI(3,4,5)P3 is generated via phosphorylation of PI(4,5)P2 by PI3-Kinase, the products of the enzymatic reaction compete with biotinylated I(1,3,4,5)P4 for the interaction with the PI(3,4,5)P3 detector protein. In the absence of this interaction, proximity of the Donor and Acceptor beads is decreased, producing a loss of luminescent signal which is inversely related to enzyme activity. Please read Assay Note #1.

Assay Kit Notes

1. The luminescent signal produced by the AlphaScreen™ assay is dependent on the release of singlet oxygen by the Donor beads and transfer to the Acceptor beads. Thus, chemicals which act as singlet oxygen scavengers can affect the assay by quenching the production of luminescence. These include, but are not necessarily limited to: transition metals (Fe2+, Fe3+, Ni2+, Cu2+, Zn2+, Al2+), azide and ascorbic acid). The presence of these compounds in enzyme preparations or buffers can interfere with the determination of enzyme activity using the AlphaScreen™ assay.
2. Do not use the 10x Buffer provided in the AlphaScreen™ GST detection kit. Use Detection Buffer as described in 'Buffers'.
3. Final DTT concentration has an effect on AlphaScreen™ signals. For best result, limit final DTT concentration in detection mixture (25 µL) within the range of 0.4 to 2 mM. If DTT is already present in Reaction Buffer, it may not be needed in the Detection Buffer.
4. It is recommended to limit DMSO concentration at or below 10% in a compound working solution (4x conc.). DMSO concentrations at or below 2.5% in a PI3-K reaction and 1% in detection have negligible effect on AlphaScreen™ Assay. It is also recommended that test compound(s) be pre-incubated with the enzyme for 15-20 minutes prior to addition of PIP2 substrate.
5. PI3-K enzyme titration is recommended before test compounds are screened. If use human PI3-Kα enzyme (E-2000) from Echelon, an enzyme concentration of 0.5 ng/µL in a 10 µL PI3-K reaction is suggested as a starting point.
6. Recommended PIP2 substrate concentration in a 10 µL reaction is 5 µM for all PI3-K isoforms if 1x KBZ/2 mM DTT/25 µM ATP is used as Reaction Buffer. Prepare a 20 µM PIP2 substrate working solution (4x conc.) by diluting the 1 mM stock solution of PIP2 50-fold in the Reaction Buffer.
7. For PI3-Kγ isoform using the second PI3-K Reaction Buffer recipe provided, recommended substrate concentration in the reaction is 20 µM. Prepare a 80 µM PIP2 substrate working solution (4x conc.) by diluting the 1 mM stock solution of PIP2 12.5-fold in the Reaction Buffer.
8. It is recommended to set up a PIP2 substrate control reaction without PI3-K enzyme to measure the PIP2 competition in the assay, especially when a high PIP2 substrate concentration (> 5 µM in a 10 µL reaction) is used.
9. The following measures can be taken to boost PI3-K activity and PIP3 production if desired:
    a. Increase ATP concentration (up to 100 µM).
    b. Optimize PI3-K enzyme concentration.
    c. Increase Reaction time.
    d. Increase PIP2 substrate concentration in reaction (See Assay Note #8).
    e. Run PI3-K reactions at 37 °C.
10. K-1301L, K-1302L, and K-1305L are lyophilized and may appear empty.

Buffers

PI3-K Reaction Buffer (PI3-Kα) 5 mM Hepes pH 7.4, 2.5 mM MgCl2, supplement with 25 µM ATP prior to use.

PI3-K Reaction Buffer (PI3-Kα and γ) 20 mM Tris pH 7.4, 4 mM MgCl2, 10 mM NaCl, supplement with 25 µM ATP prior to use.

PI3-K Reaction Buffer (PI3-Kβ and γ) 5x KBZ Buffer, a 5x concentrated PI3-K reaction buffer, is available with catalog # K-KBZ. Prepare a fresh reaction buffer by diluting 5x KBZ buffer 5-fold in ddH2O and supplement with 2 mM DTT and 25 uM ATP prior to use. This reaction buffer has been tested with p110α/p85α, p110β/p85α, and p110γ in AlphaScreen assay showing increased enzyme activity over other reaction buffers.

Detection Buffer 10 mM Tris, pH 7.4, 150 mM NaCl, 7.5 mM EDTA, 0.1% Tween-20, supplement with 1 mM DTT prior to use (Please read Assay Notes #2 and #3).

Reagents

PI(4,5)P2 substrate (K-1301L) Tube contains 100 nmol of lyophilized diC8 PI(4,5)P2 (PIP2). Prior to the first experiment, reconstitute the lipid in 100 uL ddH2O for a stock solution of 1 mM. Vortex to fully reconstitute the lipid then centrifuge the tube. Store the 1 mM stock solution at -20 °C after use, stable for at least 6 months. Prior to each experiment, Prepare required amount of substrate working solution for that day by diluting into the appropriate Reaction Buffer. Keep working solution at room temperature (RT) prior to use. Do not reuse solution on subsequent days after diluting into Reaction Buffer.

PI(3,4,5)P3 standard (K-1302L) Tube contains 0.5 nmol of lyophilized diC8 PI(3,4,5)P3 (PIP3). Prior to the first experiment, reconstitute the lipid in 20 µL ddH2O for a stock of 25 µM. Vortex to fully reconstitute the lipid then centrifuge the tube. Store the 25 µM stock solution at -20 °C after use, stable for at least 6 months. Prior to each experiment, prepare the required amount of standard solutions for that day by diluting into the appropriate Reaction Buffer. Keep working solution at RT prior to use. Do not reuse solution on subsequent days after diluting into Reaction Buffer.

Biotinylated I(1,3,4,5)P4 (K-1305L) Tube contains 0.15 nmol of lyophilized biotin-I(1,3,4,5)P4 (B-IP4). Prior to the first experiment,

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reconstitute the reagent in 150 µL ddH2O for a stock solution of 1 µM. Vortex to fully reconstitute the b-IP4 then centrifuge the tube. Store the 1 uM stock solution at -20 °C after use, stable for at least 6 months. Prior to each experiment, prepare the required amount of B-IP4 solution for that day by diluting 20-fold in Detection Buffer for a 50 nM working solution. Keep working solution on ice prior to use. Do not reuse solution on subsequent days after diluting into Detection Buffer.

PI(3,4,5)P3 Detector (K-1303) Tube contains 5 µg of PIP3 detector. Prior to the first experiment, reconstitute in 135 µL ddH2O for a 37 µg/mL stock solution. Do not vortex the PIP3 detector. Pipet up and down along the wall to collect all the detector protein. Centrifuge the tube and leave on ice. Store the 37 µg/mL stock solution at -20 °C after use, stable for 1 month. Prior to each experiment, prepare the required amount of PIP3 detector solution for that day by diluting 20-fold in Detection Buffer for a 1.85 µg/mL working solution. Keep working solution on ice prior to use. Do not reuse solution on subsequent days after diluting into Detection Buffer.

AlphaScreen™ GST detection kit **Must be purchased separately from PerkinElmer**. Concentration for both Donor Beads and Acceptor Beads provided by PerkinElmer is 5 mg/mL. Store beads at 4 °C in the dark. Prior to each experiment, prepare the required amount of beads mixture solution for that day by diluting both beads 50-fold in Detection Buffer for a 100 µg/mL working solution. Keep working solution protected against light. Do not reuse solution on subsequent days after diluting into Detection Buffer. **IMPORTANT: The beads are light-sensitive, and should only be handled in a darkened room under subdued light (less than 100 lux). Exposure to direct light will cause a decrease in the luminescent signal produced.**

Setting Up Standard Curve

You may want to run a PIP3 standard curve before running any enzyme reactions. It may also be helpful to set up a standard curve of PIP3 alongside enzyme reactions to allow determination of the amount of PIP3 produced.

From the 25 µM stock of PIP3, dilute 10-fold in Reaction Buffer for a 2.5 µM working solution. From the 2.5 µM solution, make seven 3-fold serial dilutions into Reaction Buffer to generate a standard curve of PIP3 ranging from 1 µM to 0.46 nM final concentration in detection mixture, as outlined in Table 1 below. Also set up a standard containing 10 µL Reaction Buffer without PIP3 as a 'no competitor' control. The Table below outlines the concentrations of standard solutions, and the final PIP3 concentrations and amounts per well.

The standard curve is set up directly in the wells of a 384-well white OptiplateTM. Final volume per point is 25 µL.

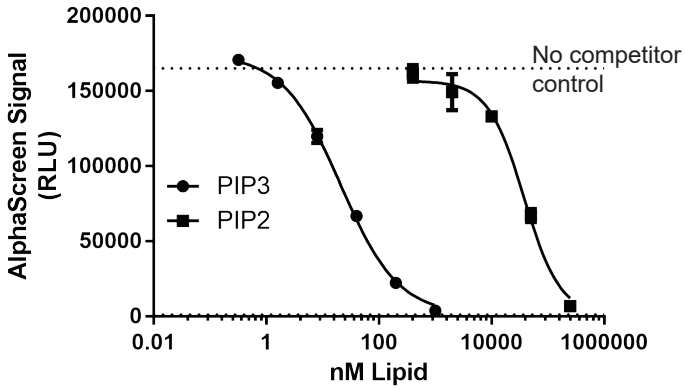
Table 1. PIP3 Standards

Concentration of PIP3 Standard, nM	Final Concentration of PIP3, nM	PIP3 per Well, pmol
2500 (2.5 mM)	1000 (1 mM)	25
833	333.2	8.3
278	111	2.8
92.6	37	0.9
30.9	12.3.	0.3
10.3	4.1	0.1
3.4	1.37	0.033
1.1	0.46	0.011
0 (no competitor)	0	0

1. Add 10 µL of each standard solution into wells.
2. Add 5 µL of 50 nM b- IP4 working solution prepared in Detection Buffer to each well.
3. Add 5 µL of 1.85 µg/mL PIP3 Detector prepared in Detection Buffer to each well.
4. Add 5 µL of Donor/Acceptor beads working solution (100 µg/mL each) prepared in Detection Buffer. Final concentration of both Donor and Acceptor beads will be 20 µg/mL.
5. Seal the plate, and incubate in a dark location at room temperature (22-27 °C) for 2 hours
6. Read plate using appropriate instrument (AlphaQuest™, Envision™, or Fusion™ Universal Microplate reader from PerkinElmer). The instrument manufacturer's suggested count times are 1 second/well with 300 msec excitation and 700 msec emission times. The competition of PIP3 standards for the interaction of the PIP3 Detector and B-IP4 bound Acceptor and Donor beads will cause a decrease in the luminescent signal which is inversely proportional to the amount of PIP3 standard.

Representative standard curves of PIP3 and PI(4,5)P2 substrate (Sigmoidal dose-response, variable slope) are shown below.

PIP Standard Curves Demonstrating Assay Specificity



Protocol for Determination of PI3-Kinase Activity

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

The entire procedure, including enzymatic reaction and detection, is performed directly in the wells of a 384-well white OptiplateTM. The final volume per assay point is 25 µL.

1. Add 2.5 µL of test compound working solution (4x conc.) to each well. See Assay Note #4.
2. Add 5 µL of purified PI3-K Enzyme (2x conc.) prepared in Reaction Buffer to each well. See Assay Note #5.
3. Add 2.5 µL of PIP2 substrate working solution (4x conc.) prepared in Reaction Buffer to each well. See Assay Notes #6, #7, and #8.
4. Seal plate and incubate at room temperature (22-27 °C) to allow enzymatic reactions to proceed, usually 1- 2 hours, without shaking.
5. When reaction is complete, add 5 µL of 50 nM B-IP4 working solution prepared in Detection Buffer.
6. Add 5 µL of 1.85 µg/mL PIP3 Detector working solution prepared in Detection Buffer. Kinase reaction is quenched at this step.
7. Add 5 µL of Donor/Acceptor beads working solution (100 µg/mL each) prepared in Detection Buffer. REMINDER: The beads are light-sensitive, and should only be handled in a darkened room under subdued light (less than 100 lux). Exposure to direct light will cause a decrease in the luminescent signal produced.

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8. Seal the plate, and incubate in a dark location at room temperature (22-27 °C) for 2 hours.
9. Read plate in AlphaScreen™ mode.

Refer to the table below for a quick guide to assay setup and optional controls.

Table 2, AlphaScreen™ Assay Quick Reference Guide

Reaction or Control	PI3-Kinase Reaction (10 µL)					PIP_3 Detection (25 µL total)				
	RB	PIP_3	Comp.	PI3-K	PIP_2	DB	B-GST	Detector	B-IP_4	Beads
Working Solution Conc.	(1x)	(1x)	(4x)	(2x)	(4x)		50 nM	1.85 µg/mL	50 nM	100 µg/mL
PIP_3 Standard	-	10 µL	-	-	-	-	-	5 µL	5 µL	5 µL
No Competitor control	10 µL	-	-	-	-	-	-	5 µL	5 µL	5 µL
Substrate control	7.5 µL	-	-	-	2.5 µL	-	-	5 µL	5 µL	5 µL
No Substrate control	5 µL	-	-	5 µL	-	-	-	5 µL	5 µL	5 µL
Enzyme Reaction control	2.5 µL	-	-	5 µL	2.5 µL	-	-	5 µL	5 µL	5 µL
Reaction w/Compound	-	-	2.5 µL	5 µL	2.5 µL	-	-	5 µL	5 µL	5 µL
Compound Background	5 µL	-	2.5 µL	-	2.5 µL	-		5 µL	5 µL	5 µL
Beads Positive control	10 µL	-	-	-	-	5 µL	5 µL	-	-	5 µL
Beads Background control	10 µL	-	-	-	-	10 µL	-	-	-	5 µL

Nomenclature: RB = Reaction Buffer; Comp. = Test Compound; DB = Detection Buffer; B-GST = biotin-GST, 500 nM stock in the AlphaScreen™ GST Detection kit, dilute 10-fold in DB prior to use.

References

1. Bohnacker T, Prota AE, Beaufils F, Burke JE, Melone A, Inglis AJ, et al. (2017) Deconvolution of Buparlisib's mechanism of action defines specific PI3K and tubulin inhibitors for therapeutic intervention. Nature Communications. 8:14683.

2. D'Angelo ND, Kim T-S, Andrews K, Booker SK, Caenepeel S, Chen K, et al. (2011) Discovery and Optimization of a Series of Benzothiazole Phosphoinositide 3-Kinase (PI3K)/Mammalian Target of Rapamycin (mTOR) Dual Inhibitors. Journal of Medicinal Chemistry. 54(6):1789-811

3. Drees B.E., Weipert A., Hudson H., Ferguson C. G., Chakravarty L. and Prestwich G. D., (2003) Competitive Fluorescence Polarization Assays for the Detection of Phosphoinositide Kinase and Phosphatase Activity,Combinatorial Chemistry & High Throughput Screening, 6(4): 321-330

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

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