Echelon Biosciences Inc.

PI(4)P 5-Kinase (PIP5KI) Activity Assay

K-5700 (96 tests)

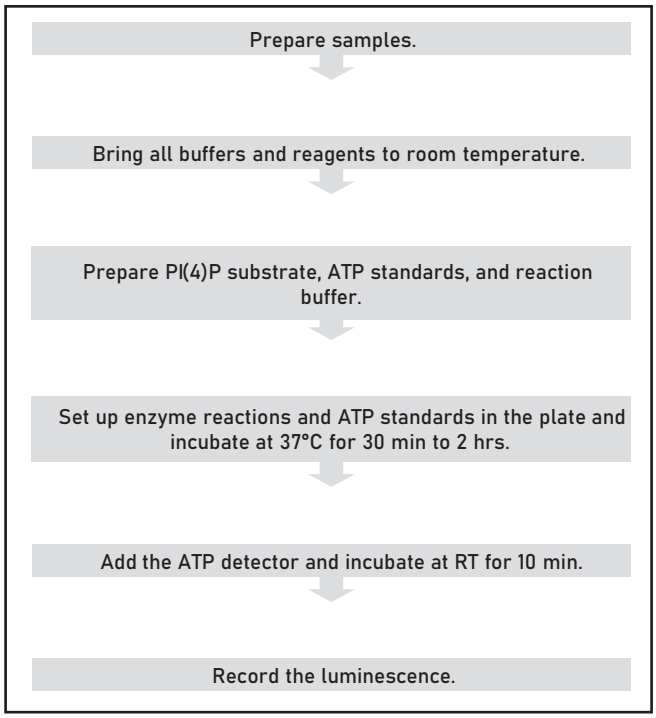
Support: echelon@echelon-inc.com

Description: ATP depletion assay for the quantification of PI(4)P 5-kinase *in vitro* or in cell lysate.

Materials Provided

Catalog #	Description	Quantity
K-5701	PI(4)P Substrate (522 μmol)	1 vial
K-KBZ	5x KBZ Buffer (10 mL)	1 bottle
K-5403	Diluent (150 μL)	1 vial
K-CF01	Kinase Cofactor (100 μL)	1 vial
K-LUMa	ATP Detector (5 mL)	1 bottle
K-ATP1	10 mM ATP (50 μL)	1 vial
K-DTT1	50 μmol dried dithiothreitol (DTT) powder	1 vial
---	96-well round bottom white plate	1 each
---	Microtiter Plate Seal	1 each

Quick Protocol



Additional Materials Provided by User:

- Source of purified PI(4)P 5-Kinase as positive control (optional)
- Plate reader capable of reading luminescence in 96-well microtiter plates
- Microcentrifuge tubes (0.5 mL or 1.5 mL) or reservoir for standard dilution or sample loading
- Multichannel pipettes or automatic pipetting station

Storage: Upon receipt, the kit should be stored at -20°C. Under proper storage conditions, the kit components should remain stable for at least 6 months from date of receipt. Allow the reagents to warm to room temperature before opening vials.

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Background

Type I PI(4)P 5-Kinase (PIP5KI) catalyzes the phosphorylation of phosphatidylinositol-4-phosphate [PI(4)P] to phosphatidylinositol-4,5-bisphosphate [PI(4,5)P2]. PIP5KI has been shown to play a role in several cellular functions including gene transcription, actin cytoskeleton reorganization, secretion and endocytosis. It is thought that PIP5KI activity is responsible for the majority of the PI(4,5)P2 found in the cell which is an important second messenger for many cellular processes.

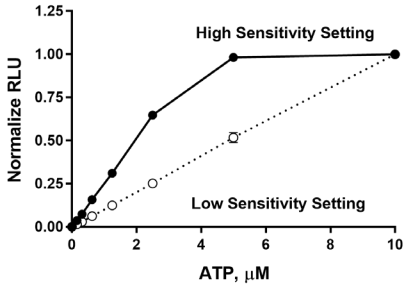
Assay Design

Echelon's PI(4)P 5-Kinase Activity Assay is an ATP depletion assay which quantifies the remaining ATP levels in solution following the kinase reaction. The assay reaction is prepared by the addition of PIP5KI (enzyme), PI(4)P (substrate) in reaction buffer and initiated by the addition of ATP. The reaction is then stopped by adding the ATP detector after the chosen reaction time. The luminescent signal is inversely correlated with the kinase activity. Echelon's PI(4)P 5-Kinase Activity Assay is tested using SignalChem's PIP5KIA (P16-10AG) kinase.

Assay Kit Notes

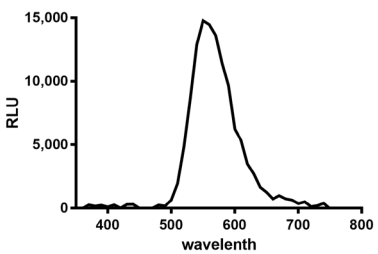
1. The ATP detector's (K-LUMa) linear range of detection may vary between instruments. Depending on the instrument capacity and sensitivity setting, the optimal range and sensitivity for the ATP detector (K-LUMa) is up to 100 µM (Figure 1).

Figure 1 | Detection System Linearity



2. The luminescent signal, or light, collected by your plate reader is not restricted to a particular wavelength. When setting up your instrument, select the option on your reader that allows the instrument to collect all wavelengths. For most plate readers this setting is labeled "all wavelengths". For BMG Labtech plate readers the "lens" setting will read all wavelengths. Please refer to your plate reader's manual and/or the manufacturer of your reader if you have questions on how to set up your specific instrument. If your instrument does not have this setting, or is limited to a certain filter, choose a broad filter centered at 550 nm (Figure 2). When the luminescent signal is read at one wavelength, a lower signal may be observed.

Figure 2 I Wavelength Emission Spectra of ATP detector; K-LUMa



3. The buffer of your sample may interfere with the luminescent output. Sample buffers should always be included in the ATP standard curve and as a "buffer only" background control.
4. Two commonly used lysis buffers (figure 3) and common lysis buffer components (figure 4) were tested in the assay. Since some buffers and lysis buffer components affect the assay, we suggest using RIPA lysis buffer. Alternatively cells can be lysed with sonication and freeze thaw cycles in the complete reaction buffer (step 3 of assay protocol). If you plan to use your own lysis buffer, we suggest testing it in the assay for interference before running samples. If your lysis buffer has a strong effect on the assay, substitute lysis buffer or dilute sample with complete reaction buffer.

Figure 3 | Lysis Buffer Effects

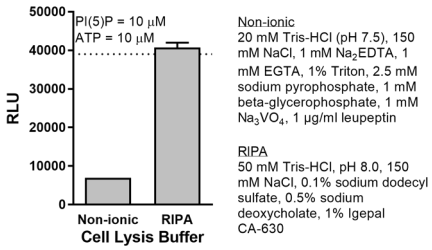
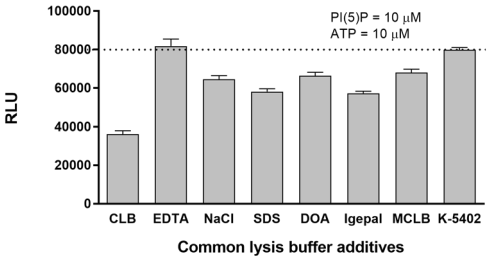


Figure 4 I Lysis Buffer Effects



5. Optimization of the kinase reactions by enzyme concentration, reaction time and temperature may be required.
6. Kinase reactions can be run at either 37°C or at room temperature. Reactions run at 37°C should be allowed to equilibrate to room temperature before the ATP detector is added to avoid a temperature gradient.
7. The provided 5X KBZ Buffer (K-KBZ) and supplements (DTT, Kinase Cofactor) must be used for the enzyme reactions.
8. The amount of kinase to use per reaction will vary according to your individual experiment. Whether you are using purified enzyme or cell lysate, you will need to test reactions using different amounts of enzyme to determine the optimum conditions. When using purified PIP5KI from SignalChem (Cat# P16-10AG), enzyme concentrations of 0.5-2 ng/µL are suggested as a starting point. In testing, we found that 5-10 µg of cell lysate was sufficient to detect significant kinase activity.
9. Sample Reaction: 10 µL samples (PIP5KI) + 10 µL PI(4)P + 20 µL ATP = 40 µL reaction.
   ATP Standards: 10 µL complete reaction buffer or sample buffer of your choice + 10 µL PI(4)P + 20 µL ATP standard dilution = 40 µL solution.

Assay Protocol

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

1. Bring all reagents to room temperature before use. Place enzyme samples, and ATP on ice until use. All steps are performed at room temperature unless noted.

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2. Add 50 µL deionized water to 50 µmol dried DTT (K-DTT1). Vortex to mix. This is the 1 M DTT stock. Keep on ice. The 1 M DTT stock is stable for at least 1 month when stored at -20 °C.
3. Prepare the complete reaction buffer by diluting the 5X KBZ Buffer (K-KBZ) to 1X and supplement with 1:1000 dilution of DTT stock and 1:500 dilution of Kinase Cofactor. Dilute only what is needed. The 1X KBZ buffer, DTT and Kinase Cofactor are not stable as complete reaction buffer.
4. Prepare a 5 mM stock of PI(4)P Substrate by adding 105 µL of the provided diluent (K-5403) to the vial of PI(4)P Substrate (K-5701). Vortex solution for a full two minutes followed by a 5 minute incubation at 37°C to fully resuspend the lipid. From the 5 mM stock prepare a 4X PI(4)P working solution by diluting the 5 mM PI(4)P stock in the complete reaction buffer (step 3). Suggested PI(4)P concentration is 100 µM (4X at 400 µM).
5. Prepare a 2X ATP solution by diluting the 10 mM ATP stock (K-ATP1) in the complete reaction buffer (step 3). Suggested ATP concentration is 10 µM (2X at 20 µM).
6. Prepare a 2-fold dilution series of ATP standards by diluting 10 mM ATP stock (K-ATP1) in 0.5 mL centrifuge tubes. A sample of a 10 µM ATP 2X dilution standard curve is shown below (Table 1).
7. Using the provided 96-well plate, add 10 µL of the 4X PI(4)P solution per well. Then, add 10 µL of the kinase samples per well. For the ATP standard wells, add 10 µL of the complete reaction buffer or sample buffer. Suggested plate layout in duplicates is shown below (Table 2).
8. Start the reaction by adding 20 µL of the 2X ATP solution to the kinase sample wells. Add 20 µL of the 2-fold ATP dilution series (prepared in step 6) to the standard wells. Cover with plate seal. Mix briefly by tapping the plate or by plate shaker. Incubate the reaction at 37°C. Incubation times may vary depending on the PIP5KI activity within samples. Suggested incubation time – 30 minutes to 2 hours. Note – The last 10 minutes of the incubation should be done at room temperature to allow the plate temperature to equilibrate.
9. After incubation, add 40 µL of ATP Detector (K-LUMa) per well. Mix briefly by tapping the plate or by plate shaker. Incubate at room temperature for at least 10 minutes to stabilize the luminescent signal. Protect from light.

Data Analysis

Kinase activity can be determined by quantifying the remaining ATP levels in the reaction buffer. An example of an ATP standard curve and PIP5KI titration are shown below.

Figure 5 | 10 µM ATP Standard Curve

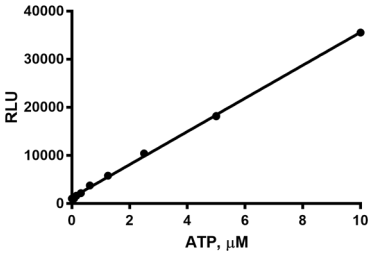

Figure 6 | PIP5KI Titration

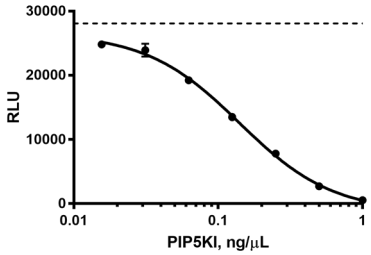

Table 1

Final ATP Conc. in Reaction (1X)	2X ATP Solutions	ATP stock (K-ATP1) or previous dilution	Complete Reaction Buffer (step 3)
10 µM	20 µM	1 µL of K-ATP1	499 µL
5 µM	10 µM	100 µL of 20µM soln.	100 µL
2.5 µM	5 µM	100 µL of 10µM soln.	100 µL
1.25 µM	2.5 µM	100 µL of 5µM soln.	100 µL
0.625 µM	1.25 µM	100 µL of 2.5µM soln.	100 µL
0.3125 µM	0.625 µM	100 µL of 1.25µM soln.	100 µL
0.15625 µM	0.3125 µM	100 µL of 0.625µM soln.	100 µL

Table 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	10 µM ATP	10 µM ATP	*Sample 1*	*Sample 1*	*Sample 9*	*Sample 9*	*Sample 17*	*Sample 17*	*Sample 25*	*Sample 25*	*Sample 33*	*Sample 33*
B	5 µM ATP	5 µM ATP	*Sample 2*	*Sample 2*	*Sample 10*	*Sample 10*	*Sample 18*	*Sample 18*	*Sample 26*	*Sample 26*	*Sample 34*	*Sample 34*
C	2.5 µM ATP	2.5 µM ATP	*Sample 3*	*Sample 3*	*Sample 11*	*Sample 11*	*Sample 19*	*Sample 19*	*Sample 27*	*Sample 27*	*Sample 35*	*Sample 35*
D	1.25 µM ATP	1.25 µM ATP	*Sample 4*	*Sample 4*	*Sample 12*	*Sample 12*	*Sample 20*	*Sample 20*	*Sample 28*	*Sample 28*	*Sample 36*	*Sample 36*
E	0.63 µM ATP	0.63 µM ATP	*Sample 5*	*Sample 5*	*Sample 13*	*Sample 13*	*Sample 21*	*Sample 21*	*Sample 29*	*Sample 29*	*Sample 37*	*Sample 37*
F	0.31 µM ATP	0.31 µM ATP	*Sample 6*	*Sample 6*	*Sample 14*	*Sample 14*	*Sample 22*	*Sample 22*	*Sample 30*	*Sample 30*	*Sample 38*	*Sample 38*
G	0.16 µM ATP	0.16 µM ATP	*Sample 7*	*Sample 7*	*Sample 15*	*Sample 15*	*Sample 23*	*Sample 23*	*Sample 31*	*Sample 31*	*Sample 39*	*Sample 39*
H	0 µM ATP	0 µM ATP	*Sample 8*	*Sample 8*	*Sample 16*	*Sample 16*	*Sample 24*	*Sample 24*	*Sample 32*	*Sample 32*	*Sample 40*	*Sample 40*

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Support Protocol

Cell Lysate Preparation

The following support protocol for cell lysate preparation has been validated for use in the PIP5KI Activity Assay. Further optimization of this protocol and/or different protocols can be used depending on the needs and experience of the user.

Materials Needed	Company	Catalog Number
1.5 mL Centifuge Tubes	N/A	N/A
RIPA Lysis Buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Igepal CA-630, 0.5% Na deoxycholate)	Sigma	R0278
Protease Inhibitor Cocktail	Sigma	P8340
PBS	N/A	N/A

This protocol is written for a 100 mm dish of HEK 293 cells (90% confluent) for about 10-15 mg/mL of cellular protein. To maximize kinase 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.

Reagent Preparation

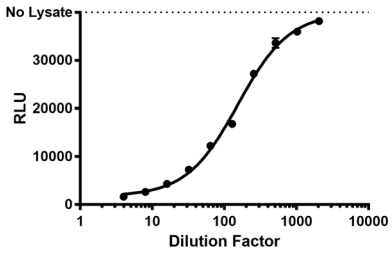
Lysis Buffer: Use a commercially available RIPA Lysis Buffer or prepare according to the recipe listed in the materials. Add the protease inhibitor cocktail fresh at a 1:100 dilution. Place buffer on ice and chill until ice cold.

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 freeze immediately at -80°C or proceed with the kinase reactions.

Note: ATP, DTT, Kinase Cofactor and PI(4)P Substrate levels should be kept consistent when setting up kinase reactions containing cell lysate. Depending on how much lysate is added to the reaction it may be necessary to supplement the lysate with these components.

Figure 7 | Cell Lysate Titration
HEK 293 Lysate, 2 Hour Incubation



Related Products

Products	Catalog Number
Subtrates	
diC16 PI(4)P	P-4016
diC8 PI(4)P	P-4008
Enzymes	
PI3Kα	E-2000
PTEN	E-3000
SHIP2	E-1000
Assays	
PI(5)P 4-Kinase Activity Assay	K-5400
PI3-Kinase Activity ELISA: Pico	K-1000s
PTEN Activity ELISA	K-4700

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