Method for Assaying the Lipid Kinase Phosphatidylinositol-5-phosphate 4-kinase α in Quantitative High-Throughput Screening (qHTS) Bioluminescent Format

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Summary

ipid kinases are important regulators of a variety of cellular processes and their dysregulation causes diseases such as cancer and metabolic diseases. Distinct lipid kinases regulate the seven different phosphorylated forms of phosphatidylinositol (PtdIns). Some lipid kinases utilize long-chain lipid substrates that have limited solubility in aqueous solutions, which can lead to difficulties in developing a robust and miniaturizable biochemical assay. The ability to prepare the lipid substrate and develop assays to identify modulators of lipid kinases is important and is the focus of this methods chapter. Herein, we describe a method to prepare a DMSO-based lipid mixture that enables the 1536-well screening of the lipid kinase phosphatidylinositol-5-phosphate 4-kinase α (PI5P4Kα) utilizing the D-myo-di16-PtIns(5)P substrate in quantitative high-throughput screening (qHTS) format using the ADP-Glo™ technology to couple the production of ADP to a bioluminescent readout.

Keywords

Quantitative high-throughput screening (qHTS); PI5P4Kα; kinase; lipid; bioluminescence; luciferase; ADP-Glo; phosphorylation

1. Introduction

Lipids are important signaling molecules that when dysregulated can contribute to human disease [1]. There are seven derivatives of phosphatidyl inositol lipids that are formed by phosphorylation at the 3-, 4-, and/or 5-positions of the inositol ring. Phosphatidylinositol-5-phosphate 4-kinases (PI5P4Ks or type II PIPKs) are lipid kinases that phosphorylate phosphatidylinositol 5-phosphate (PI-5-P), which is present in cells at very low levels [2], on the 4′ position to produce PI-4,5-P$_2$ as shown in Figure 1. There are three isoforms α, β, and γ encoded by the genes *PIP4K2A, B* and *C*. An alternate route to PI-4,5-P$_2$ is by

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phosphorylation of PI-4-P on the 5′ position by phosphatidylinositol-4-phosphate 5-kinases (PI4P5Ks or type I PIPKs), which also have three isoforms, encoded by *PIP5K1A, B,* and *C*. The type I and type II kinases have different cellular locations with the type I enzymes located at the plasma membrane and type the II enzymes localized at internal membranes. Recently, the PI5P4K α and β forms, which are upregulated in some breast cancer lines (e.g., BT474), were shown to be important for cell growth in p53-deficient breast cancer cell lines and knockdown lead to increased levels of reaction oxygen species (ROS) and induced cellular senescence [3]. Also it has been shown that the α isoform is highly expressed in acute myeloid leukemia (AML) cell lines and depletion of the α isoform by shRNA decreases cell proliferation, survival and tumorigenic activity [4].

The knockdown studies described above suggest that developing small molecule inhibitors of the PI5P4K family could be a new avenue for drug development for p53-deficient cancers with upregulated PI5P4K levels. There are a variety of assay formats that have been described previously to investigate compound modulation of kinase enzyme activity, such as HTRF KinEase (Cisbio), Transcreener FP ADP Assay (Bellbrooks), ADP-Glo (Promega)™, and transfer of γ -phosphate from radiolabelled ATP to product [5-8]. To develop a lipid kinase assay, an important consideration is the choice and preparation of the lipid substrate. Lipids are often prepared as liposomes [9], and a report utilizing the D-myo-di16-PtIns(5)P substrate, which has limited aqueous solubility, relied on commercial lipid vesicle preparation to generate 384-well assays for PI5P4K α and β that was used to screen a kinase-directed library [10]. A recent paper described a time-resolved fluorescence residence energy transfer (TR-FRET) method for assessing PI5P4Kβ activity in 384-well format utilizing the D-myo-di8-PtIns(5)P substrate, which has a shorter chain length and is soluble in assay buffer [11]. Herein, we describe a DMSO-based method with bioluminescence readout to assay PI5P4Kα activity with D-myo-di16-PtIns(5)P substrate in 1536-well format. The DMSO-based method allows the lipid mixture to be prepared directly at the bench and enabled miniaturization to the 1536-well level for a substrate with limited aqueous solubility. The assay described herein is a coupled assay in which the product ADP from the PI5P4Kα enzyme reaction is coupled through a two-step process to luminescence produced by firefly luciferase (FLuc) (ADP-Glo™), a method that has been utilized for many types of kinases [5,12].

2. Materials

Unless otherwise noted, prepare reagents using ultrapure water and store reagents at room-temperature.

2.1D-myo-di16-PtIns(5)P/DPPS Lipid Preparation

1. 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine (DPPS; Echelon Biosciences) was suspended in DMSO (1 mL DMSO per 3 mg DPPS), sonicated for 1 minute and mixed by vortexing for 30 seconds, forming a solution (see note 1).

2. D-myo-phosphatidylinositol 5 phosphate diC16 (D-myo-di16-PtIns(5)P; Echelon Biosciences) was suspended in DMSO, and alternately sonicated and mixed by

vortexing for several minutes (333 μL DMSO per 1 mg D-myo-di16-PtIns(5)P). At this stage there is still particulate matter visible.

3. 1000 μL of DPPS was added to 500 μL of D-myo-di16-PtIns(5)P making a 2:1 mixture. 2250 μL of DMSO was added, and the resulting lipid mixture was alternately sonicated and mixed by vortexing for several minutes. The result is a suspension with no visible particulate matter.

2.2 PI5P4Kα qHTS assay

1. PI5P4Kα/D-myo-di16-PtIns(5)P reagent: 10 nM PI5P4Kα, 31 μM D-myo-di16-PtIns(5)P, 79 μM DPPS, 40 mM Hepes pH 7.4, 0.25 mM EGTA, 0.1% CHAPS. Protein was expressed and purified as described in [13]. To make this reagent, 500 μL of lipid mix described in 2.1 was added to 6130 μL of buffer (43 mM Hepes pH 7.4, 0.27 mM EGTA, 0.108% CHAPS), and the mixture was sonicated and mixed by vortexing. PI5P4Kα (37 μL) was then added and the solution was gently mixed by pipetting. This reagent was stored on wet ice.

2. No PI5P4Kα buffer: 31 μM D-myo-di16-PtIns(5)P, 79 μM DPPS, 40 mM Hepes pH 7.4, 0.25 mM EGTA, 0.1% CHAPS. Assembled as described in step 1 but with 50 mM Hepes pH 7.4, 0.1% Chaps buffer replacing the enzyme.

3. No D-myo-di16-PtIns(5)P buffer: 15 nM PI5P4Kα, 40 mM Hepes pH 7.4, 0.25 mM EGTA, 0.1% CHAPS. Assembled as described in step 1 but with DMSO replacing the lipid mix (see note 2).

4. ATP buffer: 15 μM ATP, 20 mM Hepes 7.4, 60 mM $MgCl_2$, 0.1% CHAPS (see notes 3-4).

5. Thaw ADP-Glo™ Reagent (Promega) at room temperature per manufacturer's protocol (see note 5).

6. Thaw Kinase Detection Reagent (Promega) at room temperature per manufacturer's protocol and transfer kinase detection buffer to powdered kinase detection reagent making sure the buffer is fully dissolved. If precipitate is present in the buffer, the supernatant can be removed and the precipitate discarded or the solution can be warmed with swirling to 37 °C per the manufacturer's protocol prior to addition to the powdered kinase detection reagent.

7. Assay Plates: 1536-well white solid bottom medium-binding high-base plates from Greiner were used.

8. The assay reagents were dispensed using a BioRAPTR (Beckman Coulter).

9. The Lopac[1280] (Sigma-Aldrich) library was screened and the control compound was Tyrphostin AG82 (Cayman Chemical Company). Compounds were transferred to the assay plate with a pintool (Kalypsys Systems) (see note 6).

10. The assay plate was read on a Viewlux (Perkin Elmer) plate reader.

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 PI5P4Kα qHTS assay

1. Dispense 2 μL of PI5P4Kα/D-myo-di16-PtIns(5)P enzyme reagent into columns 1, 2, 5-48 of the assay plate with a BioRAPTR.

2. Dispense 2 μL of no PI5P4Kα buffer control into column 4 as the control for normalizing.

3. Dispense 2 μL of no D-myo-di16-PtIns(5)P buffer into column 3 as a control for assessment of enzyme uncoupling.

4. Centrifuge for 10 sec at 300xg.

5. Transfer 23 nL of each library compound dissolved in DMSO from a 1536-well clear compound plate arrayed in columns 5-48 and 23 nL of control compounds (DMSO in column 1, 3-4, and Tyrphostin AG82 [13] in duplicate 16-pt dose response in column 2) from a 1536-well compound plate arrayed in columns 1-4 into the assay plate using the pintool (see note 7).

6. Incubate the assay plate for 15 minutes to allow for compound binding.

7. Initiate the enzyme reaction by dispensing 1 μL of ATP buffer to all wells of the assay plate using the BioRaptr.

8. Cover the plate with a solid gasketed lid that prevents both evaporation and exposure to light.

9. Incubate at room temperature for 1 hour.

10. Dispense 2 μL of ADP-Glo™ reagent (see note 8).

11. Incubate the lidded plate for 40 minutes.

12. Dispense 4 μL of Kinase Detection reagent.

13. Incubate the lidded plate for 30 minutes.

14. Read the luminescence signal (20 sec exposure) with a ViewLux.

15. Normalize the data using the DMSO-treated control columns with (maximum signal; column 1) and without (minimum signal, column 4) enzyme. Figure 2 shows the assay statistics for a six-plate qHTS library screen of the Lopac[1280] library [13] (see notes 9-11).

4. Notes

1. Making the lipid reagents in glass vials will minimize sticking that can occur to plastic containers. The DPPS/D-myo-di16-PtIns(5)P lipid mixture can be made ahead of time and stored in glass vials at -20 °C until use. The mixture is stable to at least 6 freeze thaw cycles. The lipid mixture can be thawed at room temperature and sonicated prior to use. For the dispensing on the BioRAPTR, a glass vial was

placed inside the plastic container and the samples were placed in the glass vial. D-myo-di16-PtIns(5)P is minimally soluble in DMSO. Therefore it is recommended to add the DPPS DMSO solution to the D-myo-di16-PtIns(5)P followed by sonication to assist the formation of the uniform suspension.

2. Contaminating ATPases in the enzyme preparation can confound the ADP-Glo™ data on your lipid kinase. Testing whether the protein prep has ATPase activity in the absence of substrate can indicate a contaminating ATPase or, particularly in the case of a tyrosine kinase, some autophosphorylation activity.

3. The Ultra Pure ATP that is included in the ATP-Glo™ kit was used here. It is important to use a source of ATP that contains very low levels of ADP which will interfere with the assay.

4. The assay conditions have ATP present at the K_m which provides maximum sensitivity to identifying all three types of inhibitors (competitive, noncompetitive and uncompetitive) [14]. This assay format can be modified to determine the mechanism of inhibition with respect to ATP by running the assay at various ATP concentrations spanning 0.25 to $10 \times K_m$ of ATP [13]. Then, by plotting the $[ATP]/K_m$ vs. IC_{50}, the slope of the line will indicate the mechanism of action with respect to ATP: a positive slope indicates competitive, a negative slope indicates noncompetitive and no slope indicates uncompetitive mode of inhibition.

5. The ADP-Glo™ kit can be used per the manufacturer's protocol for concentrations of ATP up to 1 mM, and it is able to detect pmol levels of ADP. The final Mg^{2+} concentration in the reaction must be between 0.5 mM and 50 mM. The kit is able to tolerate up to 5% DMSO per the manufacturer's protocol and that is the concentration of DMSO utilized in this assay. These ADP-Glo™ reagents can be refrozen and stored at -20 °C for subsequent use. Any particulates present upon thawing should be removed prior to dispensing on the BioRAPTR.

6. Additional details for preparing the compound plates for qHTS is described in [15].

7. The tolerability of the enzyme assay to DMSO should be tested if the compounds are to be added as a DMSO solution. Here the final reaction DMSO concentration is 5% and there is no effect of DMSO until >15%.

8. In order to have the total volume of the final coupled reaction fit within the confines of the 1536-well plate (12 μL total volume to completely fill the well; <10.5 μL would be a recommended total volume) while still allowing the initial enzyme reaction to be in a volume amenable to pin transfer of compounds, the volume ratio of the ADP-Glo™ reagents was decreased (3 kinase reaction : 2 ADP-Glo™ : 4 Kinase Detection) compared to the manufacturer's protocol, which recommends 1 kinase reaction : 1 ADP-Glo™ : 2 Kinase Detection. The linearity of the kit was tested by using admixtures of ATP and ADP that total 5 μM and assessing the linearity of the kit at the decreased ratio of ADP-Glo™ reagents. There was no decrease in assay performance under the conditions described herein upon reduction of the ratio of reagents but this would need to be validated for alternate assay designs. Additionally, a standard curve can be made by testing the

various % conversion equivalents of the ATP/ADP mixtures to determine what % conversion the assay is being run at. For the PI5P4Kα under the conditions described herein, the assay is at 20% conversion. The enzyme concentration should be optimized for each new lot of PI5P4Kα.

9. The signal to background achieved with the DMSO-based method and the lipid vesicle method described in [10] were very similar [13].

10. The plate stats for a 6-plate screen executed against the Lopac^{1280} compound library had Z′=0.77, CV=9.3% and S/B= 12.6 [13]. The MSR of the IC$_{50}$ for Tyrphostin AG-82 was 1.29. Data were deposited in PubChem AID 652105, 652103, 743286 and 743285 (detection counterassay).

11. Set up mock reactions without PI5P4Kα kinase but with all other assay components present, including a mixture of ADP and ATP to mimic the kinase reaction results (i.e., 2 μM ADP and 3 μM ATP to mimic 20% conversion for a 5 μM ATP reaction), to test whether the test compounds interfere with any aspects of the detection system. The ADP-Glo™ kit includes multiple enzyme components, one of which, firefly luciferase, has been shown previously to be subject to modulation by small molecules [16,17].

Acknowledgments

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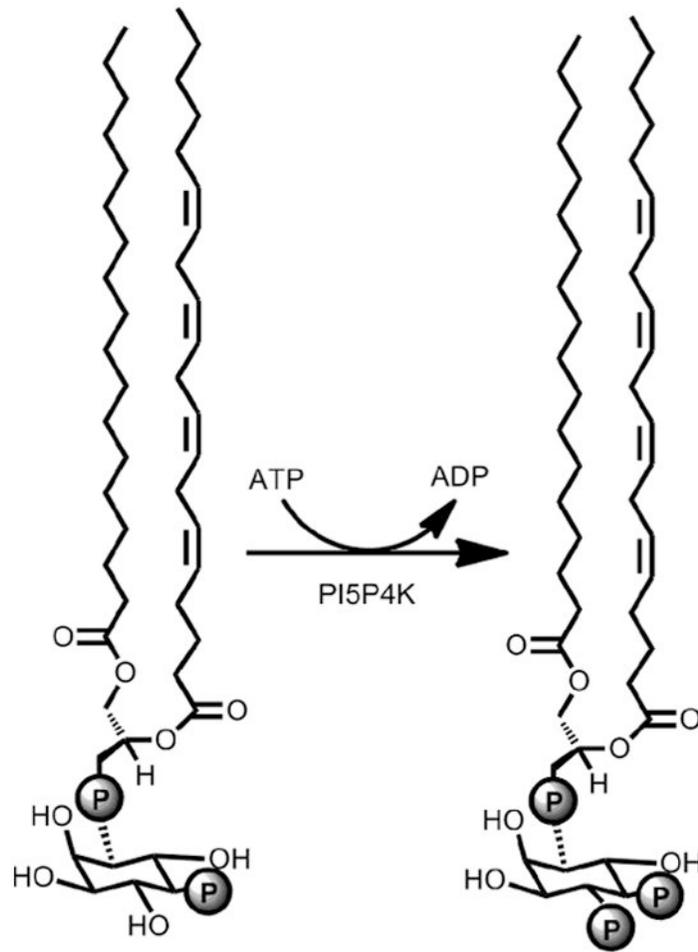
Figure 1.
Schematic representation of the phosphorylation of D-myo-di16-PtIns(5)P by the PI5P4Ks.
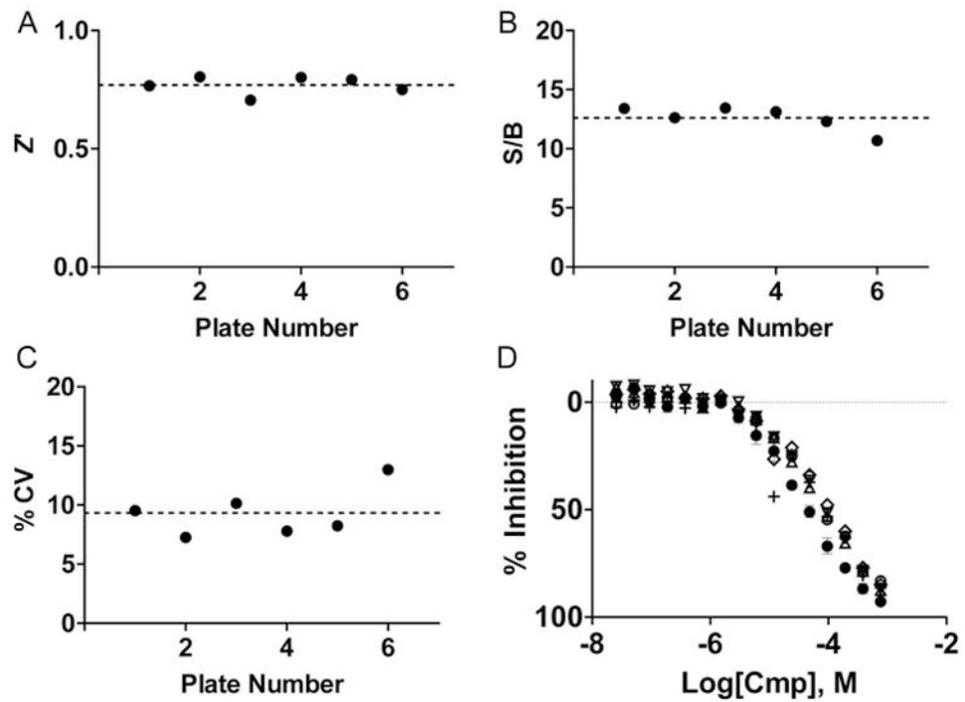Reprinted from [13] with permission from PLoS ONE.

Figure 2.
Performance of the PI5P4Kα Lopac[1280] qHTS screen in 1536-well plates. (A) Z′ factor, (B) signal/background, and (C) % column variance as a function of assay plate. (D) IC_{50} data for AG-82 control compound from each of six plates displayed with six symbols. Reprinted from [13] with permission from PLoS ONE.