TECHNICAL MANUAL

ADP-Glo™ Lipid Kinase Systems

Instructions for Use of Products
V1721, V1731, V1741, V1751, V1761, V1771, V1711, V1701, V1781, V1782, V1791, V1792, V1691 and V1690

Revised 3/20
TM365

Promega

ADP-Glo™ Lipid Kinase Systems

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

Phosphatidylinositol (PI) and its phosphorylated derivates, collectively called phosphoinositides, are important second messengers that are critical as signaling molecules and for cellular membrane remodeling (1,2). These derivatives are generated by a family of kinases called phosphoinositide lipid kinases (PIKs; Figure 1). Nineteen PIK isoforms have been identified in mammals. Based on their ability to preferentially phosphorylate the hydroxyl group of the inositol ring on position 3, 4 or 5, they have been broadly classified into three major families: phosphoinositide 3-kinases (PI3Ks), phosphoinositide 4-kinases (PI4Ks) and phosphoinositide phosphate-kinases (PIP5Ks and PIP4Ks; Figure 1 and Section 7.A; 3).

ADP-Glo™ Lipid Kinase Systems provide a complete set of reagents for performing phosphoinositide lipid kinase (PIK) reactions using a luminescent ADP-detection platform, the ADP-Glo™ Kinase Assay[a-f]. The systems include purified recombinant proteins of class I PI3Ks, optimized reaction buffer and ready-to-use lipid kinase substrates.

1. Description (continued)

Lipid substrates are supplied as frozen small unilamellar vesicles containing a mixture of phosphatidylinositol (PI) or phosphoinositol-4,5-bisphosphate (PIP2) at a 1:3 ratio with phosphatidylserine (PS) as carrier lipid. A substrate composed of PIP2 and PS at a 1:3 ratio has been optimized to use with class I PI3Ks (4). A substrate composed of PI and PS at a 1:3 ratio was demonstrated to be recognized by the majority of family members and provides a universal PI lipid kinase substrate (5,6).

The principle of the ADP-Glo™ Lipid Kinase Assay and the overview of the assay procedure are illustrated in Figures 2 and 3. The lipid kinase reaction is performed by incubating lipid substrate (PI:3PS or PIP2:3PS) with a recombinant enzyme and ATP, and the kinase activity is measured using the ADP-Glo™ Kinase Assay. The ADP-Glo™ Kinase Assay is performed in two steps. First, after the kinase reaction, an ATP-depletion reagent is added to terminate the lipid kinase reaction and deplete any remaining ATP, leaving only ADP. Second, a detection reagent is added to simultaneously convert ADP to ATP and allow the newly synthesized ATP to be converted to light using a coupled luciferase/luciferin reaction (Figures 2 and 3).

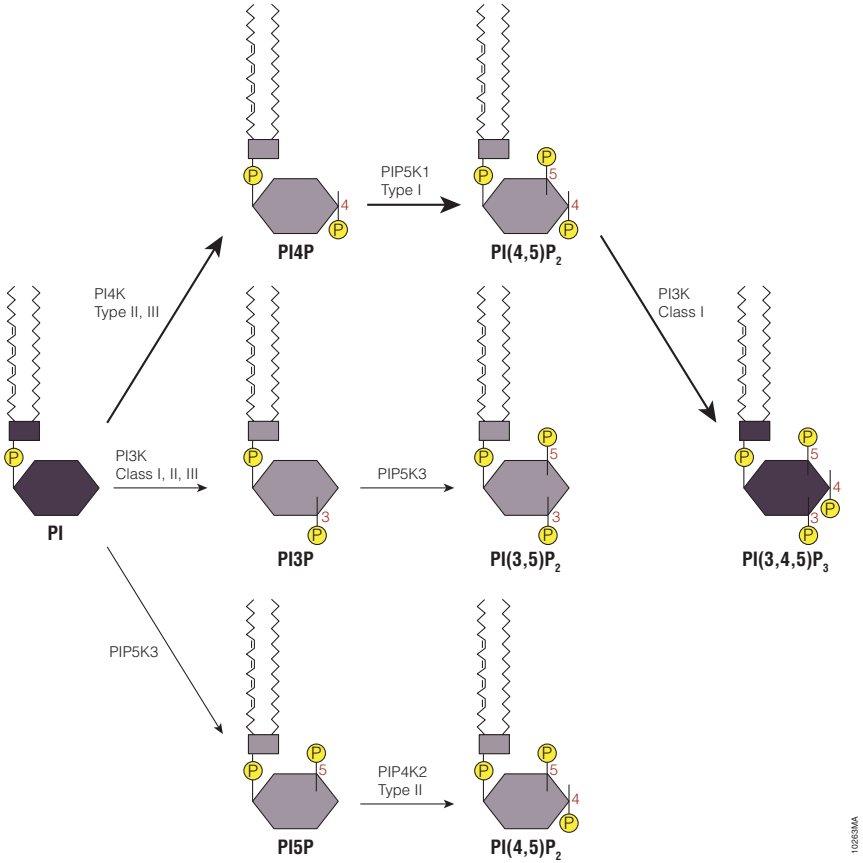


Figure 1. Reactions catalyzed by phosphoinositide lipid kinases in vitro.

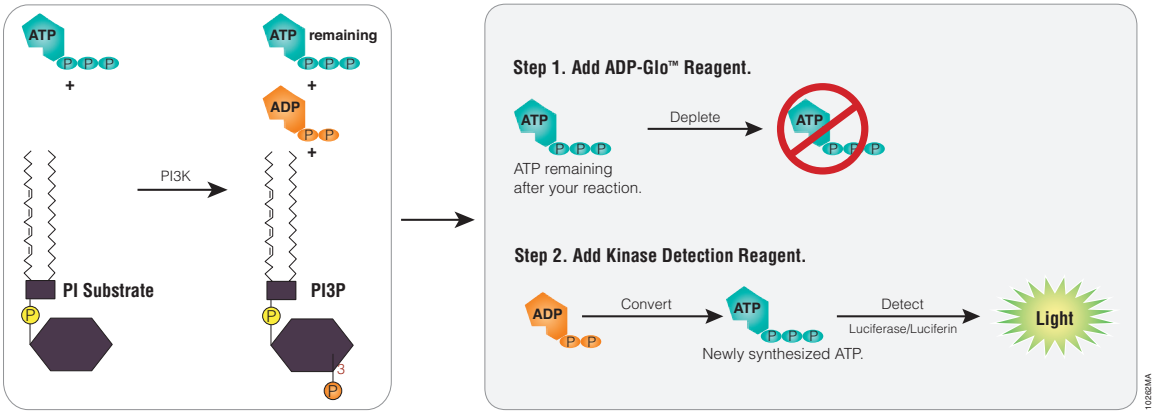


Figure 2. Principle of the ADP-Glo™ Lipid Kinase Assay. The lipid kinase reaction is performed in the presence of the appropriate substrate and ATP. Then the activity of the kinase is detected using the ADP-Glo™ Assay in two steps. First, the kinase reaction is terminated, and any ATP remaining after the reaction is depleted, leaving only ADP. Then the Kinase Detection Reagent is added to convert ADP to ATP, which is used in a coupled luciferin/luciferase reaction. The luminescent output is measured and is correlated with kinase activity.

The ADP-Glo™ Lipid Kinase Assays provide a convenient and sensitive approach for measuring activity of all classes of phosphoinositide lipid kinases (PIKs). The assays can be performed in 96- or 384-well plates and can be used for enzyme characterization, inhibitor screening or compound profiling.

Assay Advantages

- **Features:** Homogenous, robust and non-radioactive assay.
- **Flexible:** One format for lipid and protein kinases with broad linear range of ATP concentrations.
- **Positive Signal Output:** Assay signal increases linearly with increasing product formation.
- **Large Dynamic Range:** High signal-to-background ratios at lower percent conversions of ATP to ADP allow use of smaller amounts of enzyme.

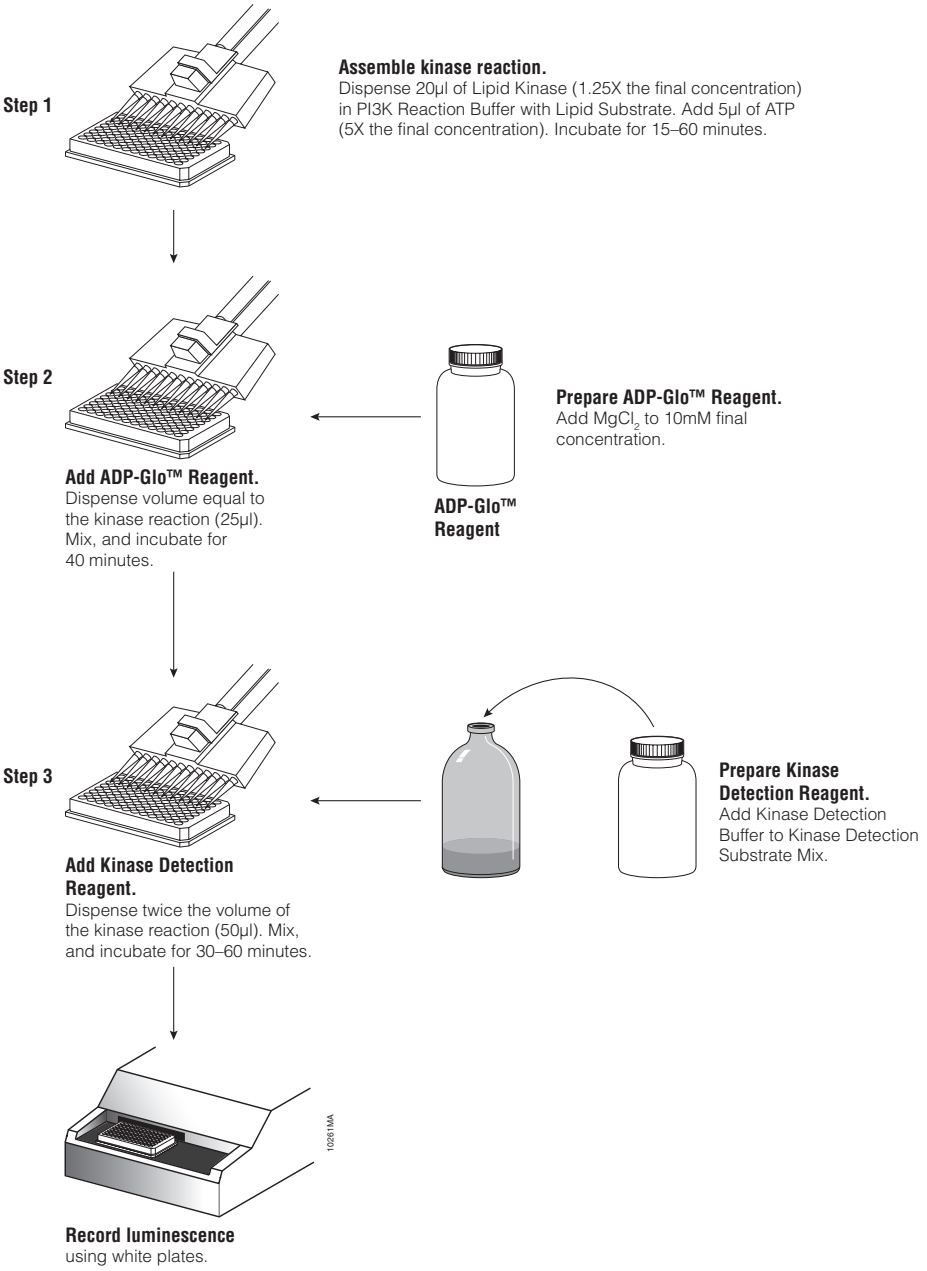


Figure 3. Schematic representation of ADP-Glo™ Lipid Kinase Assay protocol.

2. Product Components and Storage Conditions

Kinases

PRODUCT	SIZE	CAT.#
PI3K (p110α/p85α), 20µg	**200µl**	**V1721**
PI3K (p110α [E545K]/p85α), 20µg	**200µl**	**V1731**
PI3K (p110α [H1047R]/p85α), 20µg	**200µl**	**V1741**
PI3K (p110β/p85α), 20µg	**200µl**	**V1751**
PI3K (p120γ), 20µg	**200µl**	**V1761**
PI3K (p110δ/p85α), 20µg	**200µl**	**V1771**

Each recombinant enzyme is supplied with 1ml of 5X PI3K Reaction Buffer.

Lipid Substrates

PRODUCT	SIZE	CAT.#
PI:3PS Lipid Kinase Substrate, 0.5mg	**0.5ml**	**V1711**
PIP2:3PS Lipid Kinase Substrate, 0.25mg	**0.25ml**	**V1701**

V1711 and V1701 are supplied with 1ml of 10X Lipid Dilution Buffer and 1ml of 1M $MgCl_2$. The kits are sufficient for 1,000 assays if performed in 384-well LV plates using a 5µl kinase reaction or for 200 assays if performed in 96-well plates using a 25µl kinase reaction.

PI Kinase Assay Systems

PRODUCT	SIZE	CAT.#
ADP-Glo™ Kinase Assay with PI:3PS	**1,000 assays**	**V1781**

VI781 is sufficient for 1,000 assays if performed in 384-well LV plates using a 5µl kinase reaction or for 200 assays if performed in 96-well plates using a 25µl kinse reaction. Includes:

- 1 each V1711 PI:3PS Lipid Kinase Substrate, 0.5mg
- 1 each V9101 ADP-Glo™ Kinase Assay, 1,000 assays

2. Product Components and Storage Conditions (continued)

PRODUCT	SIZE	CAT.#
ADP-Glo™ Kinase Assay with PI:3PS	**10,000 assays**	**V1782**

VI782 is sufficient for 10,000 assays if performed in 384-well LV plates using a 5µl kinase reaction or for 2,000 assays if performed in 96-well plates using a 25µl kinase reaction. Includes:

- 1 each V1712 PI:3PS Lipid Kinase Substrate, 5mg
- 1 each V9102 ADP-Glo™ Kinase Assay, 10,000 assays

PRODUCT	SIZE	CAT.#
ADP-Glo™ Kinase Assay with PIP2:3PS	**1,000 assays**	**V1791**

VI791 is sufficient for 1,000 assays if performed in 384-well LV plates using a 5µl kinase reaction or for 200 assays if performed in 96-well plates using a 25µl kinase reaction. Includes:

- 1 each V1701 PIP2:3PS Lipid Kinase Substrate, 0.25mg
- 1 each V9101 ADP-Glo™ Kinase Assay, 1,000 assays

PRODUCT	SIZE	CAT.#
ADP-Glo™ Kinase Assay with PIP2:3PS	**10,000 assays**	**V1792**

VI792 is sufficient for 10,000 assays if performed in 384-well LV plates using a 5µl kinase reaction or for 2,000 assays if performed in 96-well plates using a 25µl kinase reaction. Includes:

- 1 each V1702 PIP2:3PS Lipid Kinase Substrate, 2.5mg
- 1 each V9102 ADP-Glo™ Kinase Assay, 10,000 assays

PRODUCT	SIZE	CAT.#
PI3K Class I Enzyme System	**1 each**	**V1691**

Includes:

- 50µl PI3K (p110α/p85α), 5µg
- 50µl PI3K (p110β/p85α), 5µg
- 50µl PI3K (p120γ), 5µg
- 50µl PI3K (p110δ/p85α), 5µg
- 2 × 1ml PI3K Reaction Buffer, 5X

PRODUCT	SIZE	CAT.#
PI3K-Glo™ Class I Profiling Kit	**1 each**	**V1690**

V1690 is sufficient for 1,000 assays if performed in 384-well LV plates using a 5µl kinase reaction or for 200 assays if performed in 96-well plates using a 25µl kinase reaction. Includes:

- 1 kit V1691 PI3K Class I Enzyme Kit
- 1 kit V1701 PIP2:3PS Lipid Kinase Substrate, 0.25mg
- 1 kit V9101 ADP-Glo™ Kinase Assay, 1,000 assays

Storage Conditions

Recombinant PI3K Enzymes: Store recombinant PI3K enzymes below –65°C. At first use, rapidly thaw and place on ice. Dispense any unused material into single-use aliquots and immediately snap-freeze the vials. Avoid multiple freeze-thaw cycles.

Lipid Substrates: Store lipid substrates below –65°C. Before use, thaw at room temperature and allow substrate to equilibrate completely to room temperature. Mix extensively by vortexing for at least 1 minute. Thawed lipid substrates can be kept at room temperature (15–30°C) for at least 6 hours or stored at 2–10°C for one week.

Buffers: Store 5X PI3K Reaction Buffer, 10X Lipid Dilution Buffer and 1M $MgCl_2$ at –30°C to –10°C.

ADP-Glo™ Kinase Assay: Upon receiving ADP-Glo™ Kinase Assay, remove ATP and store it below –65°C. Store the rest of the components at –30 to –10°C. Before use, thaw all components completely at room temperature. Once thawed, mix each component thoroughly before use. Because ATP is naturally prone to hydrolysis after freeze-thaw cycles, dispense into single-use aliquots and store below –65°C. Once thawed and prepared, dispense Kinase Detection Reagent (Kinase Detection Buffer + Substrate) and ADP-Glo™ Reagent into aliquots and store at –30 to –10°C. For convenience, both reagents may be used at room temperature for 24 hours without loss of signal.

3. Before You Begin

Lipid kinase assays are performed in 1X PI3K Reaction Buffer using 0.1mg/ml of PI:3PS or 0.05mg/ml of PIP2:3PS lipid substrate at 25µM ATP. The assays can be performed with other ATP concentrations in an analogous manner.

If other buffers or lipid substrates are used, their compatibility with the ADP-Glo™ detection system has to be determined. For more information about the ADP-Glo™ Kinase Assay, see the *ADP-Glo™ Kinase Assay Technical Manual* #TM313 available at: **www.promega.com/protocols/**

Materials to Be Supplied by the User

- solid-white multiwell plate
- multichannel pipet or automated pipetting station
- plate shaker
- luminometer capable of reading multiwell plates
- vortex mixer

3.A. Preparing ADP-Glo™ Reagents

Preparing ADP-Glo™ Reagent

1. Equilibrate the ADP-Glo™ Reagent to room temperature before use.
2. Transfer the volume of ADP-Glo™ Reagent required for your experiments.
3. Add $MgCl_2$ to 10mM final concentration.
4. The prepared reagent is stable for 24 hours at room temperature.
5. Dispense the remaining ADP-Glo™ Reagent into aliquots and store at −30 to −10°C.

Preparing Kinase Detection Reagent

1. Equilibrate the Kinase Detection Buffer and Kinase Detection Substrate to room temperature before use.
2. Transfer the entire volume of Kinase Detection Buffer into the amber bottle containing Kinase Detection Substrate to reconstitute the lyophilized substrate. This forms the Kinase Detection Reagent.
3. Mix by gently vortexing, swirling or inverting the contents to obtain a homogeneous solution.
4. The Kinase Detection Reagent should be used within 24 hours or dispensed into aliquots and stored at −30 to −10°C. The reconstituted reagent remains stable with no loss of signal after several freeze-thaw cycles.

Preparing 2.5X PI3K Reaction Buffer

1. Equilibrate the 5X PI3K Reaction Buffer to room temperature before use.
2. Transfer the volume of 5X PI3K Reaction Buffer required for your experiments.
3. Add an equal volume of ddH_2O.

3.B. Prepairing Lipid Kinase Substrates

Two lipid kinase substrates, PI:3PS and PIP2:3PS, were developed for use with ADP-Glo™ Lipid Kinase Assays.

PI:3PS Lipid Kinase Substrate consists of 1mg of phosphatidylinositol (PI) and 3mg of phosphatidylserine (PS) added as a carrier phospholipid. PI:3PS is a universal substrate for all members of PI3K and PI4K families. The recommended final PI:3PS concentration in a kinase reaction is 0.1mg/ml.

PIP2:3PS Lipid Kinase Substrate consists of 1mg of phosphoinositol-4,5-bisphosphate (PIP2) formulated with 3mg of PS. PIP2:3PS is a selective substrate for class I PI3Ks. The recommended final PIP2:3PS concentration in a kinase reaction is 0.05mg/ml.

Note: Before adding to a kinase reaction, the lipid substrates are prepared at 2.5X final concentration in 2.5X Lipid Dilution Buffer. The calculations are based on PI or PIP2 concentrations. Do not dilute lipid substrates directly into PI3K Reaction Buffer since lipid vesicles are unstable and start precipitating in this buffer.

3.B. Preparing Lipid Kinase Substrates (continued)

Preparation of 1ml of 2.5X Lipid Substrate working solution

1. Thaw stock solution of PI:3PS or PIP2:3PS, and equilibrate to room temperature.
2. Mix by extensive vortexing for at least 1 minute.
4. Add 250µl of PI:3PS stock solution or 125µl of PIP2:3PS stock solution to 250µl of 10X Lipid Dilution Buffer.
5. Add water up to 1ml.
6. Mix well by extensive vortexing for at least 1 minute.

   Note: Lipid substrates should be mixed well every time before use.

3.C. Creating a Standard Curve for Conversion of ATP to ADP

In order to estimate the amount of ADP produced in kinase reaction and to ensure that lipid kinase reactions are performed under initial rates of substrate to product conversion (typically 5%–10%), an ATP-to-ADP conversion curve should be performed. The conversion curves will also indicate the performance of detection reagents and can aid in instrument optimization.

Perform the ATP-to-ADP conversion curve at the desired ATP and substrate concentrations. A setup of the ATP-to-ADP conversion curve for the ADP-Glo™ Lipid Kinase Assay using 0.05mg/ml of PIP2:3PS substrate at 25µM ATP is provided. The standard curve starting at 40% conversion of ATP to ADP is shown in Figure 4.

3.C. Creating a Standard Curve for Conversion of ATP to ADP (continued)

Protocol

Part 1: Prepare master plate of ATP + ADP dilutions at 5X ATP concentration that is used in the kinase reaction. For example, if lipid kinase reactions are performed at 25µM ATP, a 125µM series of ATP + ADP standards are prepared as outlined in Table 1.

Table 1. Outline of Master Plate for Preparing ATP + ADP Dilutions at 125µM ATP.

Well Number	A1	A2	A3	A4	A5	A6
% Conversion of ATP to ADP	40% ADP + 60% ATP	20% ADP + 80% ATP	10% ADP + 90% ATP	5% ADP + 95% ATP	2.5% ADP + 97.5% ATP	no ADP + 100% ATP
125µM ADP (µl)	40	20	10	5	2.5	0
125µM ATP (µl)	60	80	90	95	97.5	100

1. Prepare 600µl of 125µM ATP and 600µl 125µM of ADP by diluting the supplied Ultra Pure ATP and ADP in water.

   Note: Use only the provided Ultra Pure ATP. Other sources of ATP may contain ADP that could result in higher background signals.

2. Combine the 125µM ATP and ADP solutions prepared in Step 1 in wells A1–A6 as indicated in Table 1 to simulate the ATP and ADP concentrations at each percent of product (ADP) formation.

3. Mix well.

Part 2: Assemble ATP + ADP conversion curve plate. Generate the curve using the lipid substrate that will be used for the kinase reactions. In the example provided, we use PIP2:3PS lipids.

1. To wells A1–A6 add 10µl of 2.5X PI3K Reaction Buffer and 10µl of 2.5X PIP2:3PS working solution prepared in Section 3.B.

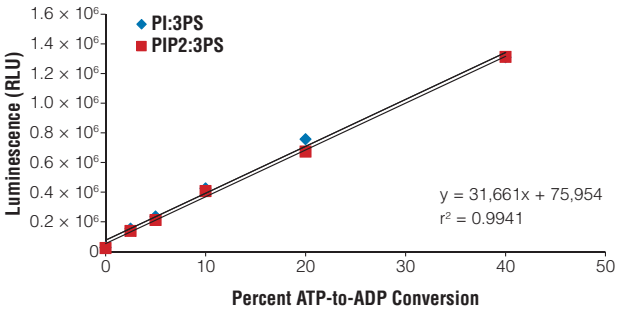
   Note: The 2.5X PI3K Reaction Buffer and 2.5X PIP2:3PS Lipid Substrates can be mixed together and 20µl of the mixture added to the wells.

2. Transfer 5µl of the ATP + ADP dilutions from the master plate (Table 1) to the corresponding wells in the assay plate (A1 to A1, A2 to A2, etc.). This is the 25µM ATP +ADP conversion curve.

   Note: Preferably, the conversion curve is done in triplicates and in the same plate as the kinase reactions.

Part 3: Follow the ADP-Glo™ Lipid Kinase Assay protocol in Section 4.A, starting at Step 7.

A.



B.

ATP = 25µM

% ADP	40	20	10	5	2.5	0
Relative Light Units (RLU)	13.12×10^5	6.73×10^5	4.06×10^5	2.11×10^5	1.38×10^5	0.23×10^5
S:B Ratio	55.5	28.5	17.2	8.9	5.8	1

10260MA

S:B Ratio = Signal-to-Background Ratio

Figure 4. Example of standard ATP-to-ADP conversion curve at 25µM ATP. ATP–to-ADP conversion curves were created as described in Section 3.C. The assay was performed in a solid-white, 96-well plate. Luminescence was recorded using a GloMax® 96 Microplate Luminometer. Values represent the mean of four replicates. Although the absolute values will vary between plate readers, the signal-to-background ratio should not be affected. **Panel A** shows a linear relationship between the luminescent signal and the amount of ADP in the reaction buffer with PI:3PS or PIP2:3PS lipid substrates. **Panel B** shows the raw RLU (Relative Light Units) values with PIP2:3PS substrate and calculated signal-to-background ratios at different percent of product formation.

4. Protocols

The protocols are provided for 25µl reactions in a 96-well plate. Other volumes may be used, provided the 1:1:2 ratio of enzyme reaction volume to ADP-Glo™ Reagent volume to Kinase Detection Reagent volume is maintained.

4.A. Optimizing Enzyme Concentration

To determine optimal enzyme concentration, set up a kinase titration experiment at the desired ATP and lipid substrate concentrations. The optimal amount of lipid kinase to be used in subsequent compound screens and test compound IC_{50} determination is the amount of Lipid Kinase Enzyme that produces luminescence within the linear range of the kinase titration curve with less than 10% substrate conversion to product. Representative data are shown in Figure 5.

1. Combine equal volumes of 2.5X PI3K Reaction Buffer and 2.5X Lipid Substrate working solution prepared in Step 3.B.
2. Make a twofold serial dilution of Lipid Kinases directly in prepared PI3K Reaction Buffer/Lipid Substrate mixture prepared in Step 1 starting at 5ng/µl.
3. Mix well.
4. Transfer 20µl from each well into a reaction plate.
5. Start reaction by adding 5µl of 125µM ATP diluted in water from supplied 10mM stock solution.
6. Cover the assay plate, mix for 30 to 60 seconds, and incubate 1 hour at 23°C (room temperature).
7. Add 25µl of ADP-Glo™ Reagent containing 10mM $MgCl_2$ prepared in Section 3.A to stop the enzyme reaction and deplete unconsumed ATP.
8. Incubate at 23°C (room temperature) for 40 minutes.
9. Add 50µl of Kinase Detection Reagent to convert ADP to ATP, and introduce luciferase and luciferin to detect ATP.
10. Incubate at 23°C (room temperature) for 40 minutes.
11. Measure the luminescence with a plate-reading luminometer or charge-coupled device (CCD) camera.

    Note: Instrument settings depend on the manufacturer. An integration time of 0.25–1 second per well should serve as a guideline. The long half-life of the ADP-Glo™ Kinase Assay signal allows plates to be left longer at room temperature before reading if desired.

    Optional: Kinase reactions can be assembled using alternative protocols. *For example:* Make a twofold serial dilution of lipid kinase in 2.5X PI3K Reaction Buffer starting from 10ng/µl. Transfer 10µl from each well into a reaction plate. Combine 2.5X Lipid Substrate working solution prepared in Section 3.B with 125µM ATP working solution diluted in water at a 2:1 ratio. Start the reaction by adding 15µl of prepared lipid substrate/ATP mixture directly to diluted kinases. Proceed to Step 6.

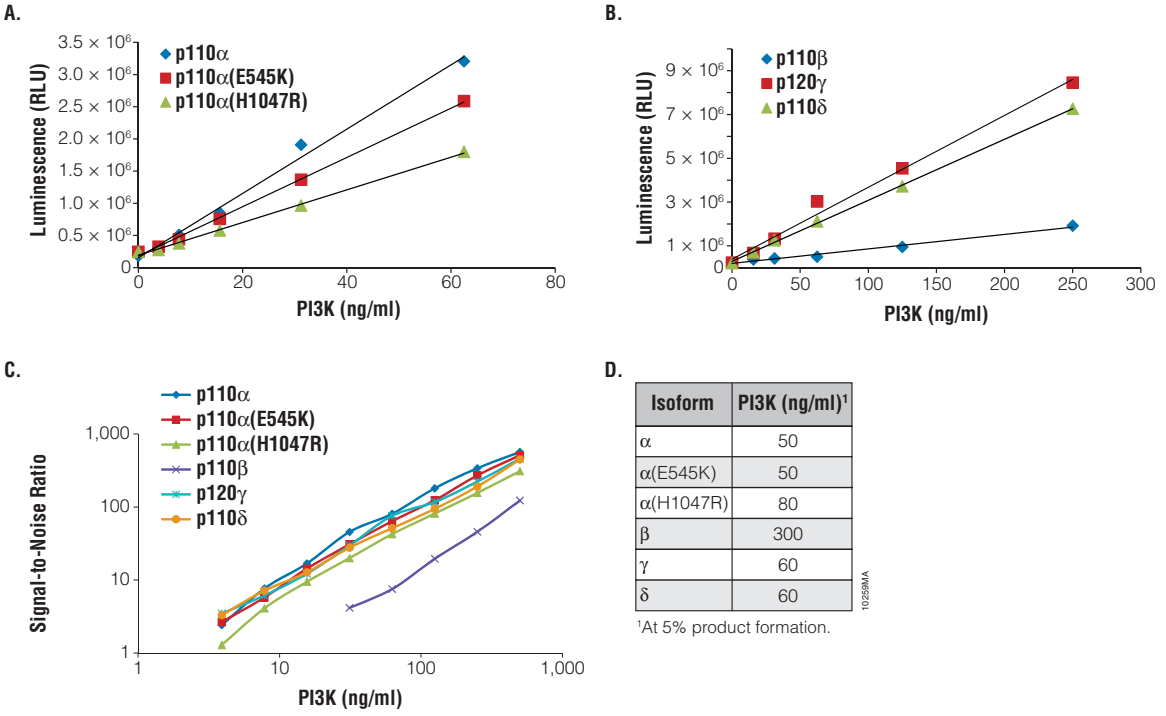


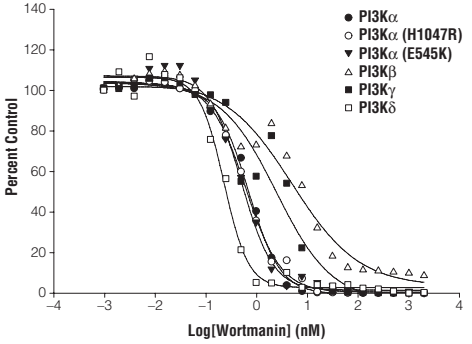
Figure 5. Examples of lipid kinase titrations. The titration of lipid kinases was performed in solid white, 96-well plates in a total volume of 25µl in PI3K kinase reaction buffer at 25µM final ATP concentration with 0.05mg/ml PIP2:3PS lipid substrates. Assays were performed at 23°C for 1 hour. **Panels A** and **B** show representative data of the enzyme titration curves. Each data point represents the mean +/− standard deviation of four samples. **Panel C** shows sensitivity and linear range of the reaction. **Panel D** shows the amount of enzyme required for 5% product formation. This represents an optimal assay window for setting up inhibitor screens since the reactions are performed under conditions of initial rates. High Z´-factor values are obtained under these conditions (6,7).

4.B. Inhibitor Titrations

The following protocol is an example of an inhibitor titration in a kinase reaction at 25µM final ATP concentration in a 25µl reaction volume; the actual volumes and ATP concentrations can be adjusted as needed. Representative inhibitor titration data are shown in Figure 6.

1. Make a twofold serial dilution of test inhibitor.
2. Transfer 2.5µl of the test compound serial dilutions to the assay plate.
3. Set up no-enzyme (background) and no-test compound (maximum signal) control wells. Add 2.5µl of test compound vehicle to those wells.
4. Prepare PI3K Reaction Buffer/Lipid Substrate mixture by combining equal volumes of 2.5X PI3K Reaction buffer with 2.5X Lipid Substrate working solution prepared in Section 3.B.
5. Dilute Lipid Kinase Enzyme into prepared PI3K Reaction Buffer/Lipid Substrate mixture at 1.25X the final desired concentration. This is your working kinase solution.
6. Mix well.
7. Transfer 20µl of prepared working kinase solution to the test compound wells except for the no-enzyme (background) control well. Add 20µl of PI3K Reaction Buffer/Lipid Substrate mixture without enzyme into the background control well.
8. Incubate at room temperature for 10–20 minutes to allow inhibitor binding to kinase.
9. Start reaction by adding 2.5µl of 250µM ATP diluted in water from supplied 10mM stock solution.
10. Mix the plate, cover the plate and incubate for the desired amount of time.
11. Follow the ADP-Glo™ Kinase Assay protocol described in Section 4.A, starting at Step 7.
12. Record luminescence.

A.



B.

Isoform	PI3K (nM)	IC_{50} (nM)
α	1.1	0.68
α(E545K)	0.79	0.6
α(H1047R)	0.61	0.5
β	5	4.9
γ	2	2.4
δ	0.8	0.2

10257MA

Figure 6. Example of general PI3K inhibitor potency data. IC_{50} values of the known PI3K inhibitor wortmannin were determined using the protocol described in Section 4.B. No-enzyme (background) control values were subtracted from all data points, and the percent inhibition was calculated relative to enzyme activities in the absence of inhibitor (100% activity). Data were plotted using the sigmoidal dose-response, variable slope model supplied with SigmaPlot 9.0 software and are shown in **Panel A**. **Panel B** shows the amount of enzyme used in the reactions and the calculated IC_{50} values for different class I PI3K isoforms.

5. General Considerations

Assay Conditions

Reaction Buffer: We recommend running the reaction for one hour at room temperature in PI3K reaction buffer (50mM HEPES [pH 7.5] 50mM NaCl, 3mM $MgCl_2$, 0.025mg/ml BSA) plus any additional additives that may be required for a specific kinase (see Table 3). If other reaction buffers are used, make sure that the $MgCl_2$ concentration is at least 5mM after adding the ADP-Glo™ Reagent to the enzyme reaction.

Lipid Kinases: While working with Lipid Kinases, we recommend avoiding freeze-thaw cycles. When preparing kinase working solution, dilute Lipid Kinases directly in assay buffer containing Lipid Substrates.

Lipid Substrates: Lipid Substrates should be diluted in Lipid Dilution Buffer (25mM HEPES [pH 7.5] 0.5mM EGTA) before combining with PI3K Reaction Buffer in order to minimize potential lipid precipitation at Mg^{2+} concentrations >5mM. Lipid Substrates are stable in Lipid Dilution Buffer for at least 6 hours at room temperature or can be stored at 2–10°C for 1 week. Lipid Substrates should be equilibrated to room temperature and properly mixed before addition to the kinase reaction. Because of the inherent lipid property to bind nonspecifically to plastics, minimize pipetting steps when working with these substrates.

Plates and Instruments: We recommend using standard solid white, multiwell plates suitable for luminescence measurements (e.g., Corning Cat.# 3912, 3674). The assay data can be recorded on a variety of plate readers; although, the relative light units will depend on the instrument. Assay well geometry and small dispensing volumes may affect the efficiency of mixing, and poor assay homogeneity in individual wells, may result in increased reaction noise and/or reduced signals. A standard ATP-to-ADP conversion curve is useful for liquid handling and instrument optimization.

Assay Controls

No-Enzyme Control: Set up wells in triplicate without an enzyme to serve as the negative control to determine background luminescence. The no-enzyme control has to be set up under the same conditions as the kinase reaction (reaction volume, buffer, lipid substrates and ATP concentration).

No-Substrate Control: Set up wells in triplicate with the optimal amount of enzyme to determine substrate-independent ATP hydrolysis. The control has to be set up under the same conditions as the kinase reaction (reaction volume, buffer and ATP concentration). Substrate-independent ATP hydrolysis can indicate enzyme autophosphorylation activity or the presence of other ATP-hydrolyzing activity in enzyme preparations.

No-Inhibitor Control: The maximum-signal control is established by adding vehicle (used to deliver test compound) to wells. In most cases, this consists of a buffer system with DMSO. ADP-Glo™ Kinase Assay is compatible with up to 5% DMSO.

Known Inhibitor Control (recommended): Set up wells in triplicate or a dilution series using a known pan or isoform-specific inhibitors as positive controls for specific inhibition of PI lipid kinases.

6. References

1. Cantley, L.C. (2002) The phosphoinositide 3-kinase pathway. *Science* **296**, 1655–7.
2. Markman, B. *et al.* (2010) Status of PI3K inhibition and biomarker development in cancer therapeutics. *Annals Oncology* **21**, 683–91.
3. Brown, J.R. and Auger, K.R. (2011) Phylogenomics of phosphoinositide lipid kinases: perspectives on the evolution of second messenger signaling and drug discovery. *BMC Evolutionary Biology* **11**:4.
4. Hawkins, P.T. *et al.* (2006) Signalling through Class I PI3Ks in mammalian cells. *Biochem. Soc. Trans.* **34**, 647–62.
5. Knight, Z.A. *et al.* (2007) A membrane capture assay for lipid kinase activity. *Nat. Protocols* **2**, 2459–66.
6. Tai, A.W., Bojireddy, N. and Balla, T. (2011) A homogenous and nonisotopic assay for phosphatidylinositol 4-kinases. *Anal. Biochem.* **417**, 97–102.
7. Li, H. *et al.* (2009) Evaluation of an antibody-free ADP detection assay: ADP-Glo. *Assay Drug Dev. Technol.* **7**, 598–605.

7. Appendix

7.A. Classification of Phosphoinositide Lipid Kinases

Table 2. Phosphoinositide Lipid Kinases (PIKs).

Class	Enzyme	Accession No.	In vivo Substrate	In vitro Substrate	Function
Phosphoinositide 3-kinases (PI3Ks)					
Class IA	PI3CA/PIKR1 (p110α/p85α)	U79143/ XM_043865	PI(4,5)P2	PI, PI(4)P, and PI(4,5)P2	Generate second messenger PI(3,4,5)P3. Regulate receptor tyrosine kinase and GPCR pathways.
	PI3CB/PIKR1 (p110β/p85α)	NM_006219/ XM_043865			
	PI3CD/PIKR1 (p110δ/p85α	NM_005026/ XM_043865			
Class IB	PI3KCG (p120γ)	AF327656	PI	PI	Generate second messenger PI(3,4,5)P3. Regulate receptor tyrosine kinase and GPCR pathways.
Class II	PIK3C2A (PI3KC2α)	NP_002636.1	PI	PI	Generate PI3P, biological role less defined.
	PIK3C2B (PI3KC2β)	NP_002637.2			
	PIK3C2G (PI3KC2γ)	BC 130277			

Table 2. Phosphoinositide Lipid Kinases (PIKs) (continued).

Class	Enzyme	Accession No.	In vivo Substrate	In vitro Substrate	Function
Phosphoinositide 3-kinases (PI3Ks; continued)					
Class III	PIK3C3 (hVPS34)	NP_002638.2	PI	PI	Associates with a hVps15; essential for vesicular traffic and autophagy.
Phosphoinositide-4-kinases (PI4Ks)					
Type II	PI42KA	NM_018425	PI	PI	Generate PI4P; catalyze the first committed step in phosphoinositide synthesis.
	PI4K2B	NM_018323			
Type III (PI3K Class IV)	PI4KA (PI4Kα)	NP_00264.1			
	PI4KB (PI4Kβ)	NP_002642.1			
Phosphoinositide Phosphate-kinases (PIPKs)					
PIP5K1 Type I	PIP5K1A	NM_001135638	PI(4)P PI(3,4)P2	PI(4)P	Preferentially phosphorylate PI(4)P; generate PI(4,5)P2.
	PIP5K1B	NM_003558			
	PIP5K1C	NM_012398			
PIP4K2 Type II	PIP4K2A	NM_0050208	PI(5)P PI(3)P	PI(5)P	Preferentially phosphorylate PI(5)P; generate PI(4,5)P2.
	PIP4K2B	NM_003559.4			
	PIP4K2C	NM_001178000			
PIP5K3 Type III	PIP5K3 (PIKfyve)	NM_001178000	PI PI(3)P	PI PI(3)P	Generate PI(5)P and PI(3,5)P2.

Promega

7.B. Determination of K_m Values

ADP-Glo™ Lipid Kinases Systems provide a complete set of reagents for performing luminescence phosphoinositide lipid kinase (PIK) assays. To facilitate experimental setup for enzyme characterization and inhibitor screening for the entire family of phosphoinositide lipid kinases (PIKs), we provide assay buffer recommendations and apparent K_m values determined using recommended assay conditions. An example of apparent K_m determination for ATP is shown in Figure 7. The determined apparent ATP K_m values for the members of PI lipid kinase family are presented in Table 3.

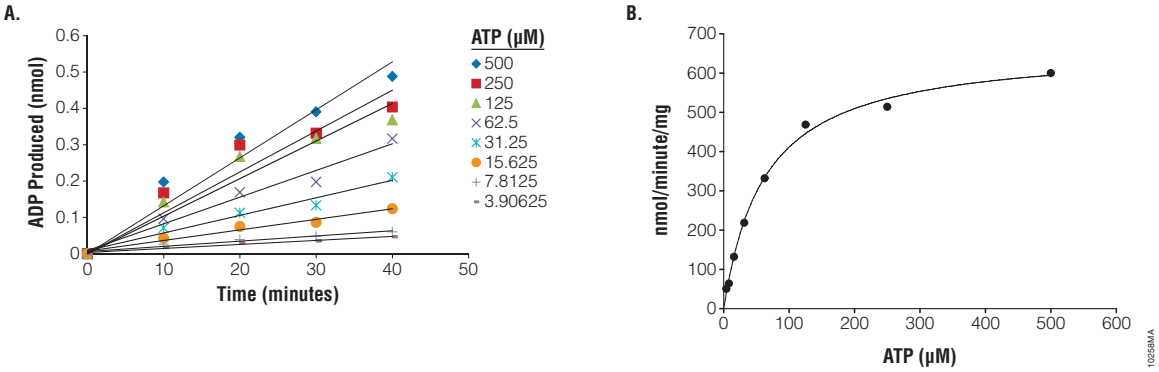


Figure 7. Example of ATP K_m apparent determination. For the ATP K_m determination, the formation of ADP was measured at different times for eight different levels of ATP. The reaction was performed at room temperature in 25µl with 22ng of PI3Kα isoform. At each ATP concentration, ATP-to-ADP conversion curves were performed and used to determine the amount of ADP produced in the kinase reaction. The produced ADP amount was plotted against the reaction time, and the initial reaction velocities were determined (**Panel A**) at each ATP concentration. Note that all the curves are linear, indicating that initial velocity conditions have been met. In **Panel B**, the initial velocity data were plotted against ATP concentration, and the apparent ATP K_m values were calculated by fitting the data to the Michaelis-Menten equation using SigmaPlot 11.0 software.

Table 3. Apparent K_m Values for ATP.

Enzyme	Substrate	Assay Buffer	ATP K_m, app (µM)	Enzyme (µg/ml)
Class I PI3Ks				
PI3CA/PIK3R1 (p110α/p85α) Cat.# V1721	0.05mg/ml PIP2:3PS Cat.# V1701	PI3K Reaction Buffer	70	0.9
PI3CB/PIK3R1 (p110β/p85α) Cat.# V1751	0.05mg/ml PIP2:3PS Cat.# V1701	PI3K Reaction Buffer	125	3.2
PI3CD/PIK3R1 (p110δ/p85α) Cat.# V1771	0.05mg/ml PIP2:3PS Cat.# V1701	PI3K Reaction Buffer	80	1.9
PI3CG (p120γ) Cat.# V1761	0.05mg/ml PIP2:3PS Cat.# V1701	PI3K Reaction Buffer	26	1.6
Class II PI3Ks				
PI3KC2A (PI3KC2α)	0.15mg/ml PI:3PS Cat.# V1711	PI3K Reaction Buffer	20	2
PI3KC2B (PI3KC2β)	0.15mg/ml PI:3PS Cat.# V1711	PI3K Reaction Buffer	50	2
PI3KC2G (PI3KC2γ)	0.15mg/ml PI:3PS Cat.# V1711	PI3K Reaction Buffer	80	1
Class III PI3Ks				
PIK3C3 (hVPS34)	0.2mg/ml PI:PS Cat.# V1711	PI3K Reaction Buffer + 5mM $MnCl_2$	40	1
PI4Ks				
PI4K2A	0.1mg/ml PI:PS Cat.# V1711	PI3K Reaction Buffer + 0.2% Triton® X-100	60	1
PI4K2B	0.1mg/ml PI:PS Cat.# V1711	PI3K Reaction Buffer + 0.2% Triton® X-100	140	1
PI4KA (PI4Kα)	0.1mg/ml PI:PS Cat.# V1711	PI3K Reaction Buffer + 0.2% Triton® X-100	70	1
PI4KB (PI4Kβ)	0.1mg/ml PI:PS Cat.# V1711	PI3K Reaction Buffer + 0.2% Triton® X-100	90	1

7.C. Calculation of Enzyme Specific Activity

Using the ADP-Glo™ Assay, the Relative Light Units (RLUs) are directly proportional to the amount of ADP, and therefore the amount of ADP produced in the kinase reaction can be directly calculated from ATP-to-ADP conversion curves. To calculate enzyme-specific activity, create enzyme titration curves as described in Section 4.A. In parallel, create ATP-to-ADP conversion curves following the protocol provided in Section 3.C. Calculate net luminescence values (RLUs) by subtracting the background values. Plot the standard curve data (amount of ADP on the X axis and RLUs on the Y axis) and perform linear regression analysis. Interpolate amount of ADP generated in the kinase reaction by comparing RLU values to the standard curve. Calculate enzyme specific activity by dividing the amount of produced ADP by reaction time and enzyme amount. The representative activity data examples are shown in Figure 8.

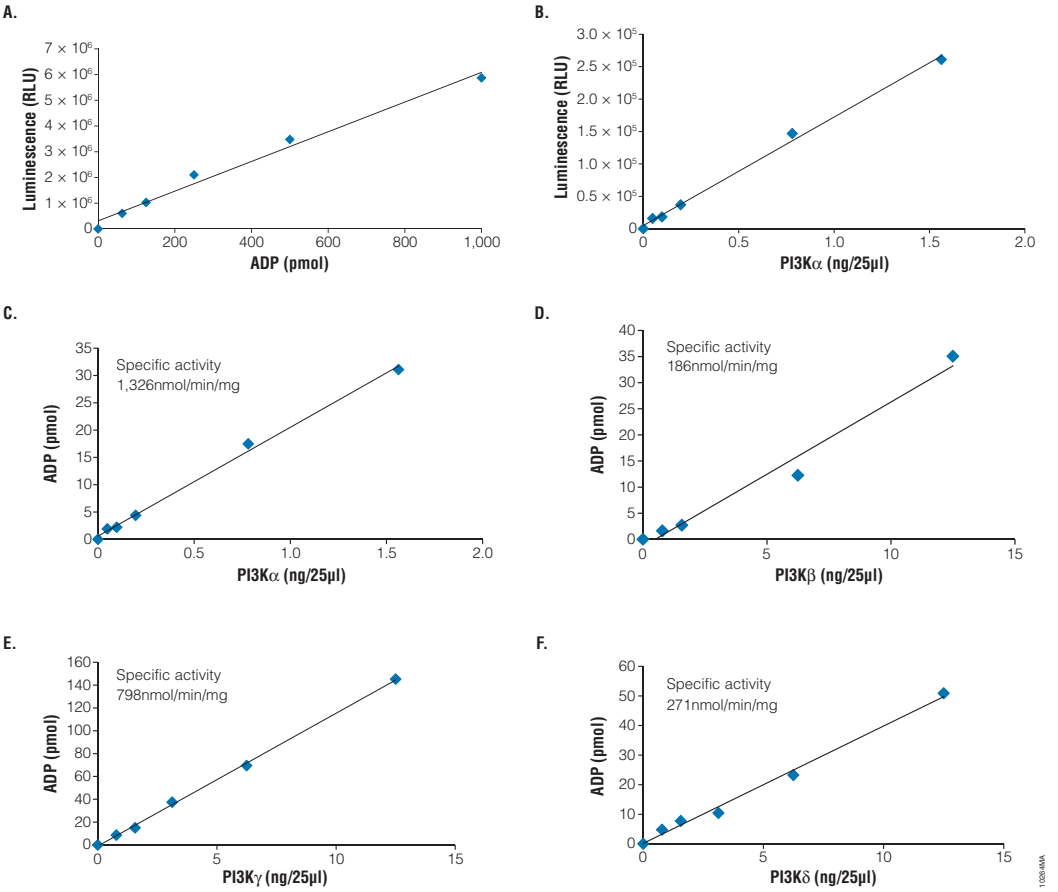


Figure 8. Examples of enzyme titration. PI3K lipid kinase reactions were performed using 0.05mg/ml PIP2:3PS lipid substrate and 100µM ATP at 30°C for 15 minutes in a 25µl reaction volume. The ATP-to-ADP calibration curve was performed in parallel with PI3K lipid kinase reactions. The ATP-to-ADP conversion data were analyzed by linear regression and are shown in **Panel A**. **Panel B** shows an example of raw data, expressed as net RLU values, for PI3Kα isoform titration. In **Panel C**, luminescent signals from the PI3Kα reaction were compared to those of the ATP-to-ADP conversion curve, and the amount of ADP produced in the lipid kinase reaction was calculated. **Panels D–F** show enzyme titration examples of β (D), γ (E) and δ (F) isoforms (luminescent signals from the reactions were compared to conversion curve, and amount of ADP produced in the lipid kinase reaction was calculated). The calculated specific activities are shown on the graphs and have been determined at a final protein concentration of 0.06µg/ml for α, 0.125µg/ml for γ and 0.5µg/ml for β and δ isoforms.

Promega

8. Related Products

ADP/ATP Detection Systems and Kinase Enzyme Systems

Product	Size	Cat.#
ADP-Glo™ Kinase Assay	400 assays	V6930
	1,000 assays	V9101
	10,000 assays	V9102
	100,000 assays	V9103
ADP-Glo™ Max Assay	1,000 assays	V7001
	10,000 assays	V7002

Promega offers **Kinase Enzyme Systems** for a number of protein kinases to help you decipher the human kinome that include enzyme, preferred substrate, buffer and other components. The Kinase Enzyme Systems are optimized for use with our ADP-Glo™ Kinase Assay and can be ordered together. The human kinome is composed of more than 500 protein kinase genes that can be grouped together based on sequence homology. The group abbreviations are as follows: **AGC**: Containing PKA, PKG, PKC families; **CAMK**: Calcium/calmodulin-dependent protein kinase; **CK1**: Casein kinase 1; **CMGC**: Containing CDK, MAPK, GSK3, CLK families; **STE**: Homologs of yeast Sterile 7, Sterile 11, Sterile 20 kinases; **TK**: Tyrosine kinase; **TKL**: Tyrosine kinase-like. For details on these systems visit: **www.promega.com/products/cell-signaling/kinase-assays-and-kinase-biology/**

Product	Size	Cat.#
Kinase-Glo® Luminescent Kinase Assay	10ml	V6711
	10 × 10ml	V6712
	100ml	V6713
	10 × 100ml	V6714
Kinase-Glo® Plus Luminescent Kinase Assay	10ml	V3771
	10 × 10ml	V3772
	100ml	V3773
	10 × 100ml	V3774
Kinase-Glo® Max Luminescent Kinase Assay	10ml	V6071
	10 × 10ml	V6072
	100ml	V6072
	10 × 100ml	V6073

Lipid Kinase Inhibitor

Product	Size	Cat.#
LY 294002	5mg	V1201

9. Summary of Changes

The following change was made to the 3/20 revision of this document:

Instructions on preparation of 2.5X PI3K Reaction Buffer were added to Section 3.A.

[a]U.S. Pat.No. 8,183,007 and other patents pending and patents pending.

[b]U.S. Pat. Nos. 6,602,677, 7,241,584 and 8,030,017, European Pat. No. 1131441, Japanese Pat. Nos. 4537573 and 4520084 and other patents pending.

[c]U.S. Pat. Nos. 7,741,067, 8,361,793, and 8,603767, Japanese Pat. No. 4485470 and other patents pending.

[d]U.S. Pat. Nos. 7,083,911, 7,452,663 and 7,732,128, European Pat. No. 1383914 and Japanese Pat. Nos. 4125600 and 4275715.

[e]U.S. Pat. No. 7,700,310, European Pat. No. 1546374 and other patents pending.

[f]Licensed from Lonza Nottingham Ltd. under U.S. Pat. Nos. 6,599,711 and 6,911,319 and other pending and issued patents.