

ASSAY VIRTUALLY ANY KINASE WITH KINASE-GLO® PLUS LUMINESCENT KINASE ASSAY, A HOMOGENEOUS HIGH-THROUGHPUT ASSAY

SAID GOUELI, PH.D., AND KEVIN HSAIO, M.S., PROMEGA CORPORATION

Phosphotransferases represent a major group of cellular enzymes that use ATP as a substrate and are implicated in a wide variety of cellular functions including proliferation, differentiation and apoptosis. Their substrates encompass a wide range of chemical structures including proteins, peptides, sugars and lipids. Because of the significance of these enzymes in cellular functions, they have become valid targets for drug development. The Kinase-Glo® Plus Assay can be used to monitor the activity of kinases and screen for kinase inhibitors. This homogeneous, non-radioactive assay is robust and amenable to high-throughput applications.

Introduction

Protein kinases play a major role in a wide variety of cellular functions and represent important targets for drug discovery (1,2). The human genome contains 518 protein kinases that are involved in the phosphorylation of 30% of cellular proteins (3). In addition, many other phosphotransferases that use ATP as substrate but are not classical protein kinases play equally important roles in cellular functions. These include inositol phosphate kinases such as phosphoinositide 3-kinases (PI3 K; 4), lipid kinases such as sphingosine kinase (5), and sugar kinases such as glucokinase (6). Screening of kinase inhibitors for the development of new therapeutics has proven successful with the FDA approval of Gleevec® protein tyrosine kinase inhibitor (STI-571) for treatment of chronic myelogenous leukemia, and ZD 1839 and OSI774 (Iressa® and erlotinib EGFR tyrosine kinase inhibitors) for non-small cell lung carcinoma. The successful outcomes of those drugs have led many pharmaceutical companies to intensify their search for more kinase inhibitors that might prove useful in the drug development (7).

Benefits of the Kinase-Glo® Plus Assay

- Use higher ATP concentrations: Linear luminescence response up to 100µM ATP.
- Use any kinase/kinase-substrate combination, including peptide, protein, lipid and sugar substrates.
- Perform the assay without substrate modifications.
- Perform batch-plate processing: Highly stable luminescent signal, with over 50% signal remaining at five hours at ambient temperature.
- Discriminate between ATP-competitive and non-competitive kinase inhibitors.
- Screen large numbers of library compounds with this fast, homogeneous non-radioactive assay.
- Obtain reliable, reproducible data: Z' -factor values routinely >0.7.

The assay can be performed with virtually any kinase and substrate combination and does not require any labeled components or antibodies.

The substrates for kinases vary from large proteins and small peptides to sugars or lipids. For some kinases that do not use protein or peptide substrates, most current protein kinase assays are not suitable for assaying their activities. Most commercially available assays use peptides as substrates, with the exception of radioactive assays that can also use proteins as substrates, and few of the current assays are capable of using sugars or lipids as substrates. We introduce the Kinase-Glo® Plus Assay^(a), which is applicable to a wide variety of substrates such as peptides, proteins, lipids and sugars. This assay uses luciferase to monitor the change in ATP levels resulting from the kinase activity. The difference in luminescence output between a negative control and the test reaction is a measure of the kinase activity. The robust

assay is homogeneous and amenable to high-throughput screening. The Kinase-Glo® Plus Assay can be used to measure activity of kinases with substrates that are prephosphorylated, such as glycogen synthase 3 kinase, or kinases that phosphorylate their substrates on multiple sites, such as IKKs.

Benefit from the Linear Response to ATP Concentration, Extended Signal Half-Life and Low Compound Interference

The principal reaction depicting the basis of the assay is shown in Figure 1. The assay can be performed in a 96-, 384- or 1536-well plate by adding an equal volume of Kinase-Glo® Plus Reagent to the completed kinase reactions and measuring luminescence. The luminescent signal correlates with the amount of ATP present as shown in Figure 2. The luminescent readout is proportional to ATP concentration up to 100µM. Because the output luminescence is a measure of the of ATP remaining in the reaction, the more active the kinase, the less luminescent signal is generated. Hence, the activity of the kinase is reciprocally correlated with luminescent output.

Universal Kinase Assay

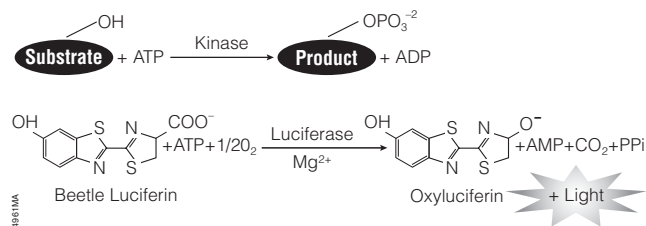


Figure 1. The Kinase-Glo® Plus Assay reaction. The kinase reaction is conducted under the appropriate conditions. ATP remaining at the time that Kinase-Glo® Plus Reagent is added is used as a substrate by the Ultra-Glo™ Luciferase to catalyze the mono-oxygenation of luciferin. The luciferase reaction produces one photon of light per turnover. Luminescence is inversely related to kinase activity.

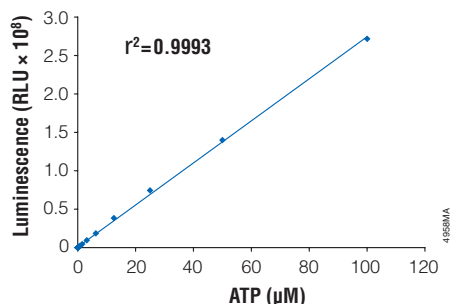


Figure 2. Luminescence correlates with amount of ATP. A direct relationship exists between the luminescence measured with the Kinase-Glo® Plus Reagent and the amount of ATP. Serial dilutions of ATP were made in a solid white, 96-well plate in 50μl of kinase reaction buffer (40mM Tris-HCl [pH 7.5], 20mM MgCl₂, and 0.1mg/ml BSA). An equal volume of Kinase-Glo® Plus Reagent was added to each well, incubated at room temperature, and luminescence was recorded using a Veritas™ Microplate Luminometer (Cat.# E6501). Values represent the mean ±S.D. of 2 replicates. There is a linear relationship between the luminescent signal and the amount of ATP in the kinase reaction buffer from 0–100μM ($r^2 = 0.9993$).

The assay can be performed with virtually any kinase and substrate combination and does not require any labeled components or antibodies. The Kinase-Glo® Plus Reagent relies on the properties of a proprietary thermostable luciferase (Ultra-Glo™ Luciferase) that is formulated to generate a stable “glow-type” luminescent signal and improve performance across a wide range of assay conditions. The signal produced by the luciferase reaction is stable, with a half-life greater than five hours (Figure 3). This extended half-life eliminates the need for a luminometer with reagent injectors and allows batch-mode processing of multiple plates. In addition, the combination of Ultra-Glo™ Luciferase and a proprietary buffer formulation result in luminescence that is much less susceptible to interference from library compounds than other luciferase-based ATP detection reagents.

Assay Virtually Any Kinase

We have tested several protein kinases using their preferred substrates in 96- and 384-well plates. The assays were carried out at 10 and 100μM ATP at room temperature and using

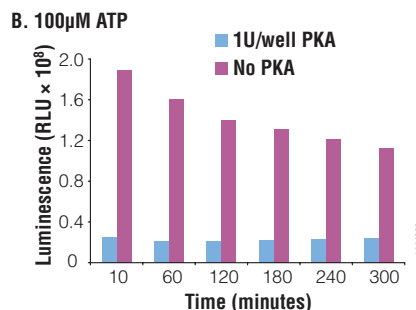
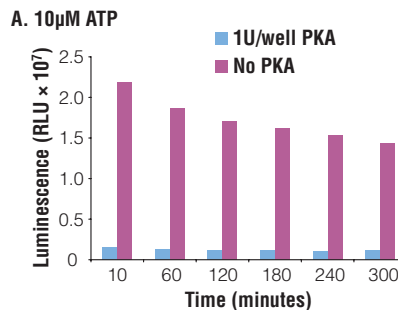


Figure 3. Extended luminescent glow-type signal. Signal stability obtained with Kinase-Glo® Plus Reagent in a solid white, 96-well plate (n = 24). PKA (Cat.# V5161) was diluted in 50μl kinase reaction buffer (40mM Tris-HCl [pH 7.5], 20mM MgCl₂, and 0.1mg/ml BSA) containing 50μM Kemptide Substrate (Cat.# V5601) and 10μM ATP (**Panel A**) or 500μM Kemptide Substrate and 100μM ATP (**Panel B**). Reactions contained one unit/well PKA (blue bars) or no PKA (red bars). Kinase-Glo® Plus Reagent was added to each well, incubated 10 minutes, and luminescence was recorded using a Veritas™ Microplate Luminometer (Cat.# E5601). Luminescence was measured at the indicated times. The signal decreased less than 25% in two hours and less than 50% in five hours. RLU = relative light units.

optimum substrate concentrations (usually three- to fivefold higher than ATP concentration). As shown in Figure 4, we have generated kinase activity profiles for a serine/threonine protein kinase (PKA, Figure 4, Panels A and B), protein tyrosine kinase (Src kinase, Figure 4, Panels C and D), and a phospholipid kinase (PI3 kinase, Figure 4, Panels E and F). The Kinase-Glo® Plus Assay can be used to monitor the activity of a broad range of kinases at different ATP concentrations.

Obtain Robust Data

An assay for high-throughput screening must demonstrate not only amenability for automation but also excellent reproducibility. A Z' factor value over 0.5 is usually considered an indicator of high performance. A value of 1.0 represents an ideal assay. The higher the value of Z' over 0.5 the better the performance of the assay (8).

$$Z' = 1 - \left[\frac{3 * S.D.^{High} + 3 * S.D.^{Low}}{Mean^{High} - Mean^{Low}} \right]$$

Using 384-well plates and either 10μM or 100μM ATP, we usually obtain Z'-factor values higher than 0.7 for the Kinase-Glo® Plus Assay (Figure 5). This high performance is required for screening a large number of compounds.

Universal Kinase Assay

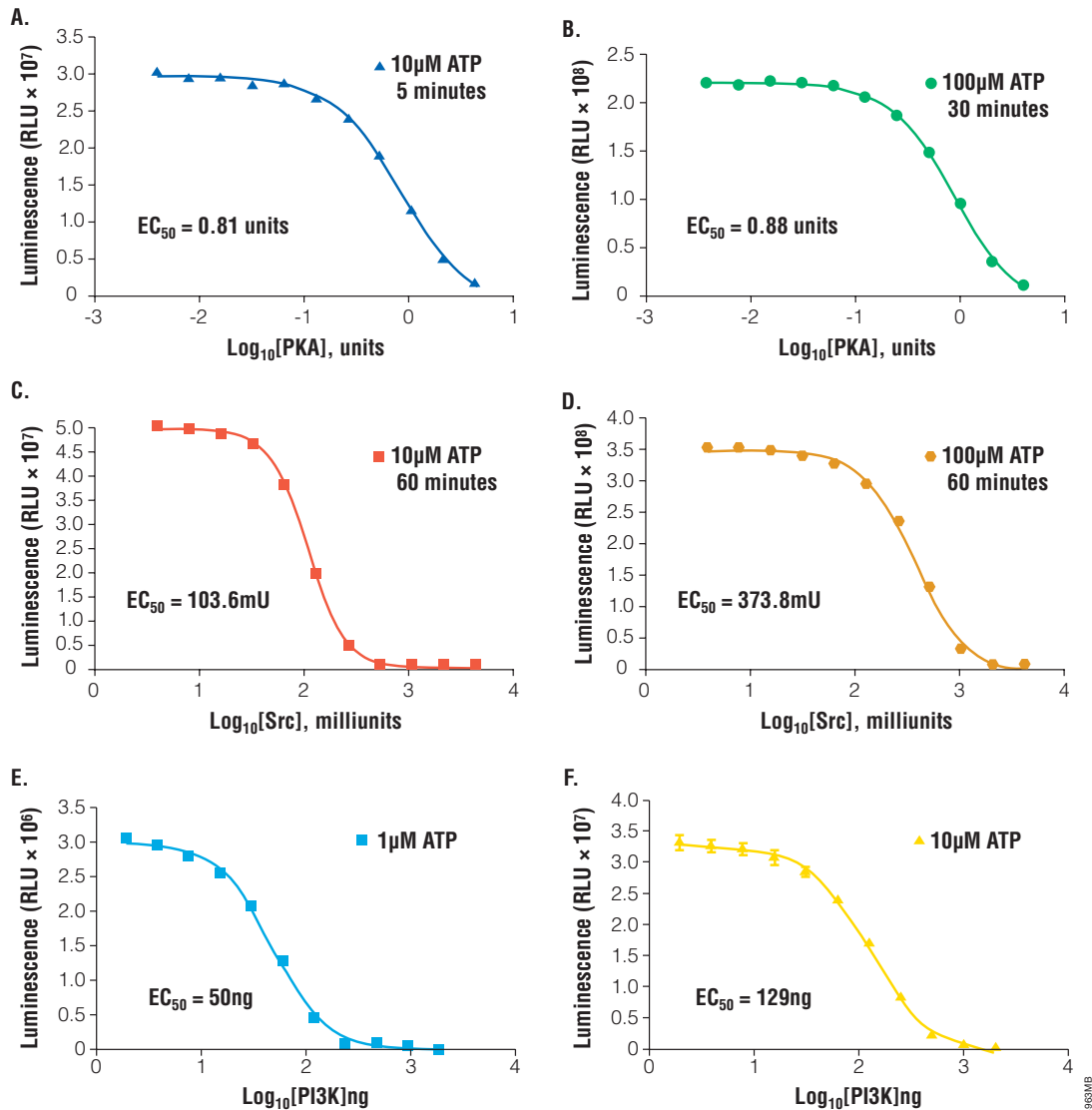


Figure 4. Luminescence is inversely correlated with kinase activity. An inverse relationship exists between luminescence measured with the Kinase-Glo® Plus Reagent and kinase activity. Serial twofold dilutions of kinase were made in solid white 96-well plates in 50 μl kinase reaction buffer under the conditions described below. Luminescence was recorded on a Veritas™ Microplate Luminometer (Cat.# E6501) ten minutes after adding an equal volume of Kinase-Glo® Plus Reagent. Curve fitting was performed using GraphPad Prism® sigmoidal dose-response (variable slope) software. **Panel A.** PKA (Cat.# V5161) was diluted in 50 μl kinase reaction buffer (40mM Tris-HCl [pH 7.5], 20mM MgCl₂, and 0.1 mg/ml BSA) containing 50 μM Kempptide Substrate (Cat.# V5601) and 10 μM ATP. The kinase reactions were run for 5 minutes at room temperature. Values represent the mean ± S.D. of two replicates. R² = 0.99; EC₅₀ = 0.81 units/well. **Panel B.** PKA (Cat.# V5161) was diluted in 50 μl kinase reaction buffer (40mM Tris [pH 7.5], 20mM MgCl₂, and 0.1 mg/ml BSA) containing 500 μM Kempptide Substrate (Cat.# V5601) and 100 μM ATP. The kinase reactions were run for 30 minutes at room temperature. Values represent the mean ± S.D. of two replicates. R₂ = 0.99; EC₅₀ = 0.88 units/well. **Panel C.** Src (Invitrogen Cat.# P3044) was diluted in 50 μl kinase reaction buffer (40mM Tris-HCl [pH 7.5], 20mM MgCl₂, 1mM MnCl₂, 0.1mM sodium orthovanadate, and 0.1 mg/ml BSA) containing 50 μM PTK peptide substrate Poly E₄Y (Sigma Cat.# P0275) and 10 μM ATP. The kinase reaction was run for 60 minutes at room temperature. Values represent the mean ± S.D. of two replicates. R² = 0.99; EC₅₀ = 373.8 milliunits/well. **Panel D.** Src (Invitrogen Cat.# P3044) was diluted in 50 μl kinase reaction buffer (40mM Tris-HCl [pH 7.5], 20mM MgCl₂, 1mM MnCl₂, 0.1mM sodium orthovanadate, and 0.1 mg/ml BSA) containing 500 μM PTK peptide substrate Poly E₄Y (Sigma Cat.# P0275) and 100 μM ATP. The kinase reaction was run for 60 minutes at room temperature. Values represent the mean ± S.D. of two replicates. R² = 0.99; EC₅₀ = 103.6 milliunits/well. **Panels E and F.** A 50 μl kinase reaction containing 10mM Tris-HCl (pH 7.5), 50mM NaCl, 5mM MgCl₂, 0.1 mg/ml BSA, and 10 μg I-α-phosphatidylinositol (Sigma Cat.# P8443) with 1 μM (**Panel E**) or 10 μM ATP (**Panel F**) was performed in a solid white 96-well plate using the indicated amount of active phosphoinositide-3-kinase p110g (Alexis Biochemicals Cat.# 201-055-C010). The reactions were mixed and allowed to incubate for 120 minutes at room temperature. Following the kinase reaction, 50 μl of Kinase-Glo® Plus Reagent was added to each well, mixed, and incubated for an additional 10 minutes at room temperature. Luminescence was read on a Veritas™ Microplate Luminometer (Cat.# E6501). All data points are the average of 2 determinations, and error bars are ± S.D.

Universal Kinase Assay

Table 1. Compounds Scored as “Hits”* in the 72-Compound Screen at 10 μ M or 100 μ M ATP.

Well #	Compound	Percent Inhibition	
		10 μ M ATP	100 μ M ATP
6	HA-1004	57.74	25.40
30	H-8	58.75	28.60
42	H-9	75.31	33.60
59	U-73122	88.19	68.60

* Hits are defined as compounds yielding results that fall more than 3 standard deviations above the mean.

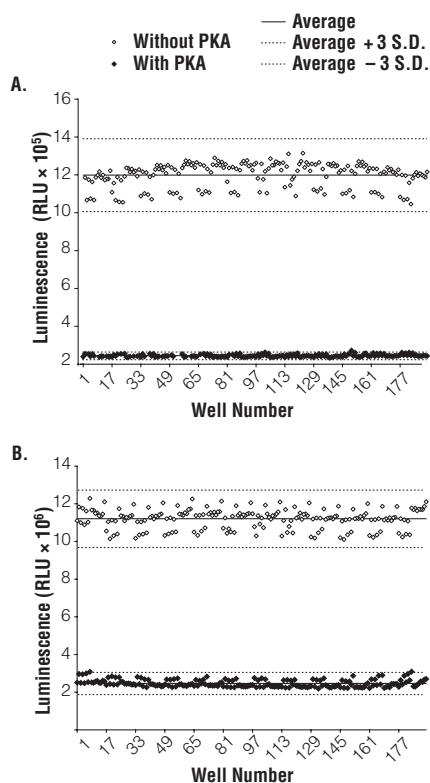


Figure 5. Determining Z'-factor in a solid white, flat-bottom 384-well plate. Panel A. The assay was performed as described in Technical Bulletin #TB343, Figure 7 with 0.2 units/well PKA and 10 μ M ATP for 5 minutes at room temperature (solid symbols) or without PKA (open symbols). Panel B. The assay was performed as described in Technical Bulletin #TB343 using 0.2 units/well PKA and 100 μ M ATP for 30 minutes at room temperature (solid symbols) or without PKA (open symbols). Final volumes of the kinase reactions for the 384-well plate assays were 20 μ l. Solid lines indicate the mean, and the dotted lines indicate \pm 3 S.D. Z'-factor values were \sim 0.8 for 10 μ M ATP and 100 μ M ATP.

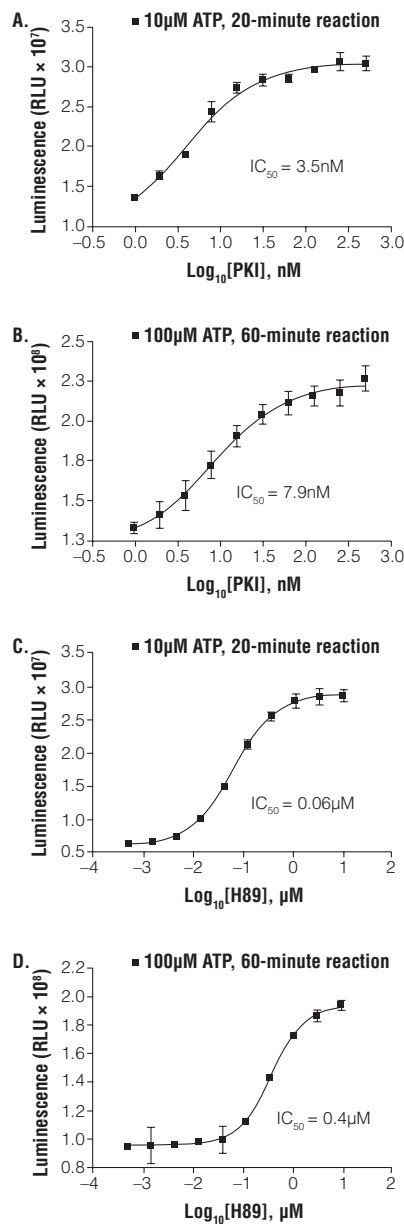


Figure 6. Determining the IC₅₀ for ATP-competitive and non-competitive inhibitors. PKA inhibitor (noncompetitive, PKI, and competitive, H89) titrations were performed in solid white, flat-bottom 96-well plates in a total volume of 50 μ l as described in Technical Bulletin #TB343, Figure 8 using 0.5 unit/well PKA and the indicated amount of inhibitor. Reactions were carried out at room temperature in 10 μ M ATP and 50 μ M peptide substrate for 20 minutes or in 100 μ M ATP and 500 μ M peptide substrate for 60 minutes. Data points are the average of two determinations, and error bars are \pm S.D. IC₅₀ results determined using the Kinase-Glo[®] Plus Assay are 3.5 nM and 7.9 nM for PKI at 10 and 100 μ M ATP, respectively. These compare favorably to the IC₅₀ values reported for these compounds in the literature [3–5 nM for PKI and 0.04 μ M for H89 at 10 μ M ATP (9,10)]. Curve fitting was performed using GraphPad Prism[®] sigmoidal dose response (variable slope) software.

Universal Kinase Assay

Screen Chemical Libraries

To validate the assay for screening chemical libraries, we used the commercially available Library of Pharmacologically Active Compounds (LOPAC; Sigma) to screen for likely inhibitors of PKA as potential hits. When we screened 640 compounds of LOPAC using PKA, we found that most hits are present in plate 6 and that most of the hits have been reported as true inhibitors of PKA (9). The data in Table 1 show that the percentage inhibition of PKA determined by the Kinase-Glo® Plus Assay at 10 and 100µM ATP. Three of the compounds showed inhibition that was dependent on ATP concentration, confirming the competitive nature of these inhibitors. For these compounds, percent inhibition of PKA is significantly lower at 100µM ATP compared to 10µM ATP. The percent inhibition of PKA by U-73122 was not significantly altered by changing ATP concentration. This compound is reported to be an inhibitor of phospholipase C.

Test ATP-Competitive and Noncompetitive Inhibitors of Protein Kinases

To demonstrate the capability of the assay in distinguishing between ATP-competitive and noncompetitive inhibitors, we selected two well known inhibitors of PKA. The compound H89 is reported in the literature as a very potent ATP-competitive inhibitor of PKA. Protein kinase inhibitor peptide (PKI) is an ATP-noncompetitive PKA inhibitor. Titration of PKI

in a 96-well plate using 0.5 units of PKA at two different ATP concentrations shows only a twofold change in IC₅₀. These values correspond to IC₅₀ values for PKI (3–5nM) reported in the literature (9). However, titration of H89 using similar assay conditions shows an almost sixfold change in IC₅₀. The lower value corresponds to the IC₅₀ (0.048µM) reported for H89 for PKA at 10µM ATP (10). These data support the notion that this assay can discriminate between ATP-competitive and noncompetitive inhibitors of protein kinases.

Conclusions

The Kinase-Glo® Plus Luminescent Kinase Assay is a homogeneous, high-throughput screening method of measuring kinase activity by quantifying the amount of ATP remaining in solution following a kinase reaction. Kinase-Glo® Plus can be used with ATP concentrations up to 100µM, which makes the assay well suited for distinguishing between ATP-competitive and noncompetitive inhibitors. The Ultra-Glo™ Luciferase and proprietary buffer result in luminescence that is less susceptible to compound interference than other ATP-based detection methods. The assay can be used with virtually any kinase/substrate combination including proteins, sugars, lipids or prephosphorylated substrates. The chemistry and design of the Kinase-Glo® Plus Assay provides users with a robust kinase assay that is amenable to high-throughput screening and is adaptable to their experimental systems. ■

Protocol

Kinase-Glo® Plus Luminescent Kinase Assay Technical Bulletin #TB343

(www.promega.com/tbs/tb343/tb343.html)

References

1. Noble, M.E.M., Endicott, J.A. and Johnson, L.N. (2004) *Science* **303**, 1800–5.
2. Vlahos, C.J., McDowell, S.A. and Clerk, A. (2003) *Nat. Rev. Drug Discov.* **2**, 99–113.
3. Manning, G. *et al.* (2002) *Trends Biochem. Sci.* **27**, 514–20.
4. Shears, S.B. (2004) *Biochem. J.* **377**, 265–80.
5. French, K.J. *et al.* (2003) *Cancer Research* **63**, 5962–9.
6. Grimsby, J. *et al.* (2003) *Science* **301**, 370–3.
7. Force, T. *et al.* (2004) *Circulation* **109**, 1196–1205.
8. Zhang, J.-H., Chung, T.D.Y. and Oldenburg, K.R. (1999) *J. Biomol. Screen.* **4**, 67–73.
9. Walsh, D.A. and Glass, D.B. (1991) *Meth. Enzymol.* **201**, 304–16.
10. Hidaka, H., Watanabe, M. and Kobayashi, K. (1991) *Meth. Enzymol.* **201**, 328–39.

Ordering Information

Product	Size	Cat. #
Kinase-Glo® Plus Luminescent Kinase Assay	10ml	V3771
	10 × 10ml	V3772
	100ml	V3773
	10 × 100ml	V3774

^(a)The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

Kinase-Glo is a registered trademark of Promega Corporation. Ultra-Glo is a trademark of Promega Corporation.

Gleevec is a registered trademark of Novartis AG Corporation. GraphPad Prism is a registered trademark of GraphPad Software, Inc. Iressa is a registered trademark of AstraZeneca UK Ltd. Veritas is a trademark of Turner BioSystems, Inc.