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 a 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).

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. 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).

The Kinase-Glo® Plus Assay can be used to measure activity of kinases and screen for kinase inhibitors. This homogeneous, non-radioactive assay is robust and amenable to high-throughput applications.

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®, 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

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 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).

\[ Z’ = 1 - [ \frac{3 \times S.D.\text{High} + 3 \times S.D.\text{Low}}{\text{MaxHigh} - \text{MinLow}} ] \]

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.
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 50µl 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 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. 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 50µl kinase reaction buffer (40mM Tris [pH 7.5], 20mM MgCl₂, and 0.1mg/ml BSA) containing 500µM Kemptide Substrate (Cat.# V5601) and 100µM 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 50µl kinase reaction buffer (40mM Tris-HCl [pH 7.5], 20mM MgCl₂, 1mM MnCl₂, 0.1mM sodium orthovanadate, and 0.1mg/ml BSA) containing 50µM PTK peptide substrate Poly E4Y (Sigma Cat.# P0275) and 10µM 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 50µl kinase reaction buffer (40mM Tris-HCl [pH 7.5], 20mM MgCl₂, 1mM MnCl₂, 0.1mM sodium orthovanadate, and 0.1mg/ml BSA) containing 500µM PTK peptide substrate Poly E4Y (Sigma Cat.# P0275) and 100µM 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 50µl kinase reaction containing 10mM Tris-HCl [pH 7.5], 50mM NaCl, 5mM MgCl₂, 0.1mg/ml BSA, and 10µg l-α-phosphatidylinositol (Sigma Cat.# P8443) with 1µM (Panel E) or 10µM 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, 50µl 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.
Table 1. Compounds Scored as “Hits”* in the 72-Compound Screen at 10µM or 100µM ATP.

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<td>59</td>
<td>U-73122</td>
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* 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 10µM 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 100µM 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 20µl. Solid lines indicate the mean, and the dotted lines indicate ±3 S.D. Z’-factor values were ~0.8 for 10µM ATP and 100µM 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 50µl 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 10µM ATP and 50µM peptide substrate for 20 minutes or in 100µM ATP and 500µM peptide substrate for 60 minutes. Data points are the average of two determinations, and error bars are ±S.D. IC50 results determined using the Kinase-Glo® Plus Assay are 3.5nM and 7.9nM for PKI at 10 and 100µM ATP, respectively. These compare favorably to the IC50 values reported for these compounds in the literature [3–5nM for PKI and 0.04µM for H89 at 10µM 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 using 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

Ordering Information

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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.

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