HIGH-THROUGHPUT KINASE SCREENING USING A UNIVERSAL LUMINESCENT KINASE ASSAY

SAID GOUELI, PH.D.[1], BRAD LARSON, B.A.[1], KEVIN HSIAO, M.S.[1], TRACY WORZELLA, M.S.[1], AOIFE GALLAGHER, PH.D.[2], AND ERIC MATTHEWS, M.S.[3], [1]PROMEGA CORPORATION, [2]DEERAC FLUIDICS, [3]BMG LABTECH

Phosphotransferases represent a major group of cellular enzymes that use ATP as a substrate and are implicated in a wide variety of cellular functions. The homogeneous Kinase-Glo® Luminescent Kinase Assay can monitor the activity of these enzymes, which have become important drug discovery targets. This non-radioactive assay is robust and easily amenable to high-throughput screening applications.

Introduction

Protein kinases play a major role in a wide variety of cellular functions and thus represent an important target for drug discovery (1,2). The human genome is reported to contain 518 protein kinases that are involved in phosphorylation of 30% of all cellular proteins (3). Additionally, many other phosphotransferases play equally important roles in cellular reactions that use ATP as a substrate but are not classified as protein kinases. These include inositol phosphate kinases such as phosphoinositide 3-kinases (PI3 kinases; 4), lipid kinases such as spingosine kinases (5) and sugar kinases such as glucokinases (6). Screening kinase inhibitors to develop new therapeutics has proven successful with the FDA approval of Gleevec (STI-571) for treating chronic myelogenous leukemia and Iressa (ZD 1839) for treating lung cancer. Many pharmaceutical companies continue to search for kinase inhibitors that might prove useful for developing novel therapeutics (7).

Using a Non-Radioactive, Universal Assay for Kinase Screening

Because the substrates for classical protein kinases vary from large proteins to small peptides to sugars or lipids, many commonly used kinase assay techniques are not suitable for universal kinase screening. The Kinase-Glo® Assay[(a,b)] is a non-radioactive universal kinase assay that can use a wide variety of substrates including peptides, proteins, lipids and sugars. The Kinase-Glo® Assay uses luciferase to monitor a decrease in ATP, which is consumed in the reaction catalyzed by the phosphotransferase (Figure 1). ATP is a universal substrate to all classes of kinases, including protein kinases. The difference in luminescent output from the Kinase-Glo® reaction following a kinase reaction is a measure of enzyme activity. The Kinase-Glo® Assay is compatible with kinases that add phosphate to a prephosphorylated substrate such as glycogen synthase 3 kinase and kinases that phosphorylate their substrates on multiple sites.

The Kinase-Glo® Assay is performed in a single tube or in 96-, 384- or 1536-well plates by adding an equal volume of Kinase-Glo® Reagent to the completed kinase reaction and measuring luminescence. The luminescent signal is proportional to the ATP concentration over at least three orders of magnitude up to 10µM ATP. Since the output luminescence is a measure of the ATP remaining in the reaction, the more active the kinase, the less luminescent signal generated. Hence, the activity of the kinase is reciprocally related to luminescence output.

The Kinase-Glo® Assay relies on the properties of a proprietary luciferase (Ultra-Glo™ Recombinant Luciferase) that generates a "glow-type" luminescent signal and performs well across a wide range of assay conditions. The Ultra-Glo™ Luciferase and the buffer formulation result in luminescence that is much less susceptible to interference from library compounds than other luciferase-based ATP detection reagents.

We demonstrate the utility of the Kinase-Glo® Assay for high-throughput screening (HTS) applications using the Deerac Fluidics Equator™ NS-808 Eight-Tip Pipetting System in low-volume 384 (LV384)- and 1536-well formats. Data obtained with these instruments are compared to those obtained manually using 96- and 384-well plate formats.

Materials and Methods

Reagents and Materials

The Kinase-Glo® Assay was performed as described in Technical Bulletin #TB318. Protein Kinase A and Kemptide substrate were purchased from Promega Corporation. All other reagents, including kinase inhibitors, were purchased from Sigma-Aldrich Corporation (St. Louis, MO). The library of phar-

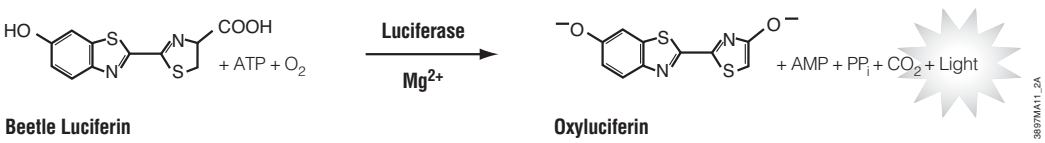

Figure 1. The luciferase reaction. Mono-oxygenation of luciferin is catalyzed by luciferase in the prescence of Mg^{2+}, ATP and molecular oxygen and produces one photon of light per turnover.

Table 1. Assay Steps and Component Volumes Dispensed in LV384- and 1536-Well Plates.

		LV384			1536		
Step	Reaction Component	10µl	6µl	3µl	8µl	5µl	2µl
1	Vehicle or Test Compound	500nl	300nl	150nl	400nl	250nl	100nl
2.	PKA	2.0µl	1.2µl	600nl	1.6µl	1.0µl	400nl
3.	Kemptide Substrate	2.5µl	1.5µl	750nl	2.0µl	1.25µl	500nl
4.	Incubate assay plate at room temperature for 20 minutes.						
5.	Kinase-Glo™ Reagent	5.0µl	3.0µl	1.5µl	4.0µl	2.5µl	1.0µl
6.	Incubate assay plate at room temperature for 20 minutes.						
7.	Read assay plate.						

macologically active compounds (LOPAC) was purchased from Sigma Aldrich Corporation. Manual assays were performed in opaque-walled 96-well plates (Costar Cat.# 3912) and read using a FLUOstar OPTIMA luminometer (BMG LABTECH; Durham, NC). Automated assays were performed using the Deerac Fluidics Equator™ NS-808 non-contact dispenser in LV384- and 1536-well plates and read using the PHERAstar multiwell luminometer (BMG LABTECH).

Optimizing Kinase Reaction Conditions

In order to obtain the maximum performance with the Kinase-Glo® Reagent, the kinase reaction conditions were optimized with respect to the amount of ATP, substrate concentration and finally enzyme concentration for screening potential kinase inhibitors. For more information please see reference 8.

Automated Non-contact HTS Liquid Dispensing

Use of high-density, low-volume plate formats for screening has created the need for pipetting systems capable of rapidly delivering compounds, enzyme-substrate mixes and reagents in a consistently in smaller and smaller volumes. We used the Deerac Fluidics Equator™ pipetting system to perform the Kinase-Glo® Assay in LV384- and 1536-well plates. This instrument was used to dispense enyzme/substrate mix, ATP and reagent to the assay plates. The assays were performed in 10, 6 and 3µl total reaction volumes in LV384-well plates and in 8, 5 and 2µl volumes in 1536-well plates (Table 1). The Equator™ pipetting system can be used with a wide range of stackers and can be integrated with other robotic systems. The volumes, concentrations and order of addition used in the automated assays are provided in Tables 1 and 2.

Applying the Kinase-Glo® Assay in Automated HTS Formats

Z′-factor analysis was used to determine the ultility of the Kinase-Glo® Assay in low-volume HTS applications. Z′-factor is a statistical measurement of the robustness of an assay; values above 0.5 are indicative of quality assays (9). We tested PKA in LV384- and 1536-well formats using optimized kinase reaction conditions. The results of the automated assays (Table 3) compare favorably to manual results previously generated in 96- and 384-well formats, showing Z′-factor values >0.8 (8).

Screening LOPAC with Kinase-Glo® Assay

We tested the Kinase-Glo® Assay using a commercially available library of compounds (LOPAC) to determine if the assay could score true kinase hits in that library. When we screened the LOPAC collection for inhibitors of PKA using the manual protocol, we found six wells in which we could detect kinase inhibition (8). The same six wells also showed detectable kinase inhibition when we tested the Kinase-Glo® Assay in the LV384- and 1536-well formats (Table 4). These results demonstrate that the automated Kinase-Glo® Assay performs well for screening large libraries for modulators of kinases in an easy and economical format. The percent inhibition values obtained with the miniaturized Kinase-Glo® Assay compare favorably with those obtained manually (Table 4).

> The Kinase-Glo® Assay is a non-radioactive universal kinase assay that can use a wide variety of substrates including peptides, proteins, lipids and sugars.

To confirm that these hits are true inhibitors of PKA, we screened the library using the ProFluor® Kinase Assay (10), which detects kinase activity based on the phosphorylation of a fluorogenic substrate. As summarized in Table 4, the same compounds were identified by the ProFluor® Assay with a similar pattern of percent inhibition, although the percent inhibition was lower. The lower values result from the fact that a different ATP concentration was used (50µM for the ProFluor® Assay rather than 10µM for the Kinase-Glo® Assay). Since these compounds are competitive inhibitors of ATP, the percent inhibition should be higher at lower concentrations of ATP. In other studies, not shown here, we have also confirmed the inhibition pattern using an assay that uses radioactive ATP (11) for phosphorylation of a peptide substrate.

Determining IC_{50} Values In Low-Volume HTS

In order to further evaluate this assay for use in HTS, we tested four known PKA inhibitors in LV384- and 1536-well formats. Test compounds were diluted manually as described in

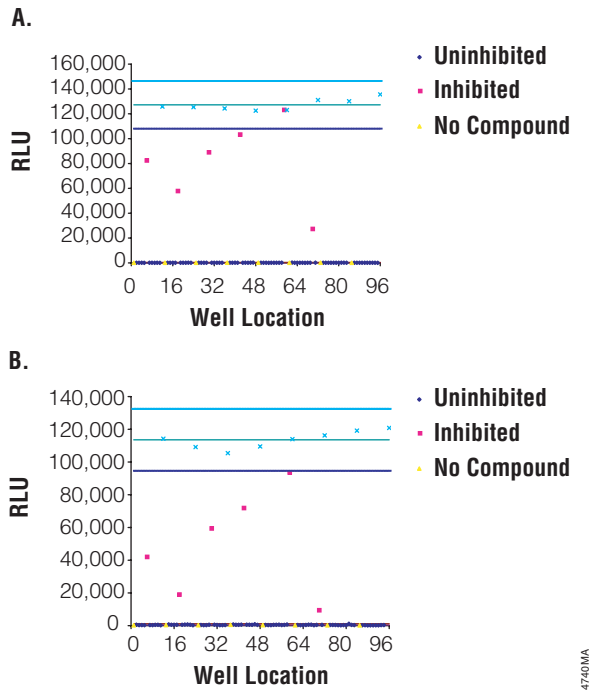

Figure 2. Compound screen using Plate 6 of LOPAC (Sigmal-RBI) performed in LV384- (Panel A) and 1536-well (Panel B) formats. Compounds were screened at 10µM in volumes described in Table 1. Percent inhibition of compounds that inhibited kinase activity are identified in Table 4.

Technical Bulletin #TB318 using 2X the optimal concentration of kinase and kinase substrate plus the appropriate vehicle concentration. The remainder of the IC_{50} assay was completed as described in Table 1 and reference 8. Certain compounds are present in the LOPAC library, while others were included because of their prevalence in the literature. The results in Figure 3 and Table 5 show that manual and automated formats of the Kinase-Glo® Assay gave IC_{50} values similar to each other as well as to values reported in the literature. This further establishes the Kinase-Glo® Assay for use in library screening of inhibitors in drug discovery laboratories.

Summary

Kinase-Glo® is a universal kinase assay that is adaptable to any combination of kinase and substrate, regardless of the nature of the substrate. The assay is non-radioactive, easy to use and can be formatted for any throughput need. It is also less influenced by background fluorescence from library test compounds and thus minimizes false hits. Excellent Z´-Factor values and accurate IC_{50} values are obtained in reaction volumes as low as 2µl. Also, the extended luminescent half-life of this reagent chemistry make this assay ideal for any high-throughput applications. ■

Table 2. Volumes and Concentrations of Components Added to the Assay Plate in LV384- or 1536-Well Plates.

Assay Format	Total Volume	Enyme Concentration
LV384	10µl	156mU
	6µl	78mU
	3µl	38mU
1536	8µl	125mU
	5µl	78mU
	2µl	31mU

Final concentrations of enzyme, substrate and ATP were determined as described in Technical Bulletin #TB318. Substrate concentration for all experiments was 31.26µM and ATP concentration was 4µM.

Table 3. Z´-Factor Values for Kinase-Glo® Assays Run on the Equator™ NS-808 Pipetting System and Read on the PHERAstar Reader.

Assay Format	Total Volume	Z´-Factor Value
LV384	10µl	0.95
	6µl	0.90
	3µl	0.83
1536	8µl	0.75
	5µl	0.74
	2µl	0.75

Table 4. Results from Screening Plate 6 of LOPAC.

		% Inhibition at 10µM			
Well #	Compound	Manual 384-well	Automated LV384-well	Automated 1536-well	ProFluor® Assay
70	GW5074	29.1	21.46	8.29	4.2
18	H-7	49.6	45.42	16.73	5.5
6	HA-1004	70.2	64.77	36.99	17.6
30	H-8	74.1	69.94	52.31	20.6
42	H-9	83.3	81.19	63.29	38.8
59	U-73122	95.7	96.74	82.38	71.9

Table 5. IC_{50} Values for PKA Inhibitors Determined Using Manual and Automated Kinase-Glo® Assays.

	IC_{50} Value		
Inhibitor	Kinase-Glo® Manual	Kinase-Glo® Automated	Literature Value
H-9	2.4	1.895	1.9
H-89	no data	0.0026	0.04
Staurosporine	0.010	0.00632	0.003

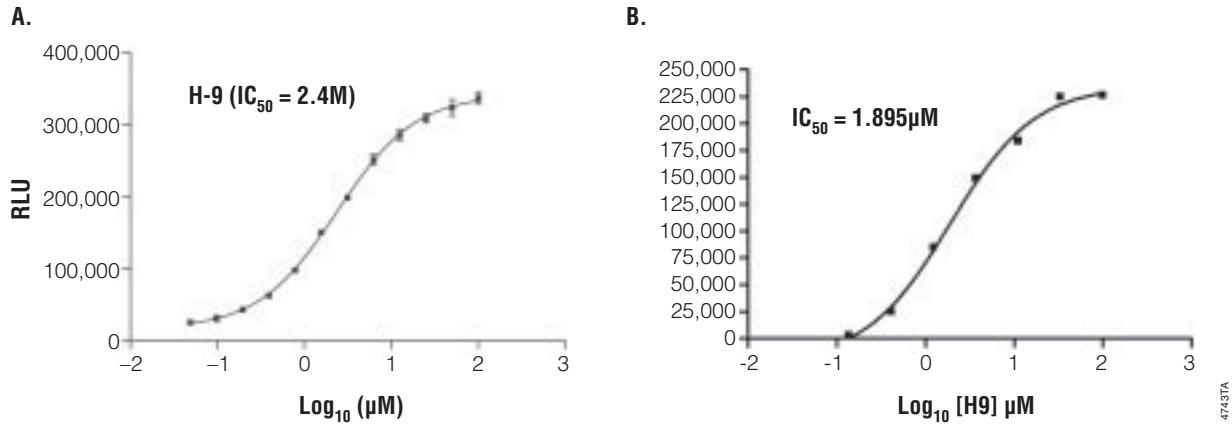

Figure 3. Titration of two hits from screening the LOPAC for PKA inhibitors. The Kinase-Glo® Assay was carried out using manual protocol in 384-well plates as described in Technical Bulletin #TB318 with varying concentrations of inhibitor H-9 (**Panel A**). **Panel B** shows the IC_{50} titration for H-9 determined using Kinase-Glo® Assay in 1536-well plates. Titration profile for H-9 IC_{50} values similar to those reported in the literature (12).

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Protocol

Kinase-Glo® Luminescent Kinase Assay Technical Bulletin #TB318
(www.promega.com/tbs/tb318/tb318.html)

Ordering Information

Product	Size	Cat.#
Kinase-Glo® Luminescent Kinase Assay[a,b]	10ml	V6711
	10 × 10ml	V6712
	100ml	V6713
	10 × 100ml	V6714

[a]U.S. Pat. No. 6,602,677, Australian Pat. No. 754312 and other patents pending.

[b]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.

[c]Patent Pending.