Optimizing Kinase Assays for Ultrahigh-Throughput Profiling Using the Kinase-Glo® Plus Assay

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Abstract

Here we demonstrate the concept of kinase profiling using a panel of four kinases and ten kinase inhibitors. We used the Kinase-Glo® Plus Luminescent Kinase Assay and the Deerac Fluidics™ Equator™ HTS non-contact liquid handling system to design and perform an ultrahigh-throughput profiling screen. We present IC_{50} values for each test compound and show how such profiling can streamline the initial drug discovery process.

Introduction

Kinases play a crucial role in regulating complex cellular processes including cell activation, growth, and differentiation. Abnormal kinase activity can lead to a variety of human diseases including cancer, arthritis, diabetes, and cardiovascular disease. The role that kinases play in these diseases makes them an attractive target class for the pharmaceutical industry (1). Pharmaceutical companies are tasked with finding potent, yet selective, kinase inhibitors to help ensure a high therapeutic effect for the target kinase with the least amount of toxicity from off-target activity.

Kinase profiling helps to determine if test compounds are selective for their intended target kinase enzymes. The profiling process involves screening compounds against a panel of kinases. Profiling can be performed using two basic strategies. The first strategy involves screening at a single compound concentration against the kinase panel. Percent inhibition for each compound with each respective kinase is calculated, and results are compared to determine which kinases are inhibited by each compound, or in other words, how selective the compound is. A second strategy for profiling involves screening the compound at several different concentrations and generating IC_{50} values for each compound with each kinase. The IC_{50} is the concentration of compound that is required to inhibit the target kinase activity by fifty percent. Profiling with IC_{50} saves a step for screeners by generating potency and selectivity data simultaneously.

In this article we demonstrate the concept of kinase profiling using a panel of four kinases and ten kinase inhibitors. The Deerac Fluidics™ Equator™ HTS non-contact liquid handling system is used for performing kinase assays in a 1536-well ultrahigh-throughput screening (uHTS) format. Ultrahigh-throughput screening is desirable for kinase profiling applications because it affords more wells for generating multiple data points. With the Equator™ system, we first optimized each kinase assay with respect to kinase amount, reaction time, and substrate concentration using the Kinase-Glo® Plus Luminescent Kinase Assay[a] (Cat.# V3771). Optimized kinase reaction conditions are then used to determine Z´-factor values for each kinase enzyme (2). Z´-factor is a statistical measure of assay variability and reproducibility (2). We profiled test compounds using the IC_{50} method of profiling, where each compound is analyzed at 24 different concentrations against each kinase. Resulting data are used to determine both selectivity and potency of each test compound against the different panel kinases.

Considerations for Optimizing Kinase Assays

Performing kinase screening first requires optimizing the reaction conditions for each kinase that will be used in the profile. In this study, we used the homogeneous Kinase-Glo® Plus Luminescent Kinase Assay. This assay determines kinase activity by quantifying the amount of ATP remaining in solution following a kinase reaction (Figure 1) and is easily amenable to uHTS formats.

Four different kinase enzymes were chosen for this application. The enzymes and their respective substrates include: PKA enzyme with kemptide substrate, Src enzyme with peptide 2 substrate, MAPKAP kinase 2 with MAPKAP substrate, and PI3 kinase with LA-phosphatidylinositol lipid substrate.

We followed three steps to optimize the kinase reactions. The first and second step, performed simultaneously, optimized both kinase reaction time and amount of kinase for each reaction. The third step optimized substrate concentration. We then determined Z´-factor values with these optimized reaction conditions to assess the quality of the assay for subsequent profiling activities.

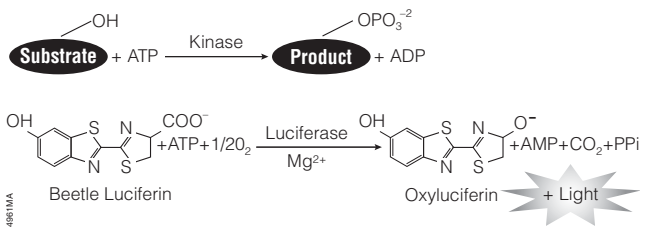

Figure 1. The Kinase-Glo® Plus Assay reactions. The kinase enzyme phosphorylates a substrate using phosphate from ATP. The more active the kinase, the more ATP is consumed. Following the kinase reaction, the amount of ATP remaining in solution is quantified with the addition of the Kinase-Glo® Plus Reagent. In the presence of ATP, luciferase catalyzes the mono-oxygenation of luciferin to generate light. Light output is inversely proportional to kinase activity.

Kinase assays were optimized at room temperature to avoid temperature gradients across the assay plates. Room temperature assays were also more amenable to high-throughput screening because they prevented a heating bottleneck in the screening process.

The Kinase-Glo® Plus Assay allows ATP concentrations up to 100µM for screening. In this profiling application, all reactions were performed with 10µM ATP. Table 1 lists the various addition steps and corresponding volumes used for each experiment described in this study.

Optimizing Reaction Time and Amount of Kinase

Substrate for each kinase was added to a 1536-well plate (Costar® Cat.# 3937). As a general guideline, we used a final substrate concentration equal to five times the amount of ATP per reaction: 50µM substrate for all kinases except PI3 kinase, where 8µg of phospholipid was used per reaction. Next, we used the gradient dispensing function of the Equator™ system to titrate enzyme by volume 1:2 across the plate with each tip dispensing a different enzyme concentration. Kinase reaction buffer was added to all wells to bring the contents to a constant volume before adding ATP to initiate reactions.

To determine optimal reaction time, Kinase-Glo® Plus Reagent was added over a series of time points to stop the reaction. The time points were selected based on the activity of the kinase being tested. Following Kinase-Glo® Plus Reagent addition, the plate was incubated for 10 minutes at room temperature and luminescence recorded with the BMG PHERAstar plate reader. Optimal kinase reaction time was defined as the point at which the EC_{50} from the enzyme titration no longer changes. The optimal kinase amount was determined from the EC_{50} curve for optimal reaction time. The optimal amount of kinase was defined as the smallest amount of enzyme that gave the largest dynamic range from the no-enzyme control in the linear portion of the titration curve (Figure 2, Table 2).

Table 1. Volumes Used in Optimization and Profiling Assays.

Optimizing Reaction Time* and Amount of Kinase	
Component	**Volume Dispensed per Well**
Substrate	2µl
Enzyme	2µl, 1µl, 0.5µl or 0.25µl
Buffer	0µl, 1µl, 1.5µl or 1.75µl
ATP	1µl
Kinase-Glo® Plus Reagent	5µl
Optimizing Substrate Concentration	
Component	**Volume Dispensed per Well**
Water	0µl, 1µl, 1.5µl or 1.75µl
Substrate	2µl, 1µl, 0.5µl or 0.25µl
Enzyme	2µl
ATP	1µl
Kinase-Glo® Plus Reagent	5µl
Determining Assay Quality with Z´-Factor Analysis	
Component	**Volume Dispensed per Well**
Water	1µl
Enzyme/Substrate	2µl
ATP	2µl
Kinase-Glo® Plus Reagent	5µl
24-Point Dose Response	
Component	**Volume Dispensed per Well**
5% DMSO	0µl, 0.5µl, 0.75µl or 0.875µl
Test Compound	1µl, 0.5µl, 0.25µl or 0.125µl
Enzyme/Substrate	2µl
ATP	2µl
Kinase-Glo® Plus Reagent	5µl

The orders of addition and corresponding volumes are provided for each type of experiment performed in this article.

*To determine optimal reaction time, Kinase-Glo® Plus Reagent was added at different time points. Each kinase has a different activity, so different time points were used for each kinase tested.

Optimizing Substrate Concentration

The optimal substrate concentration for each kinase reaction was determined using the optimal reaction time and kinase amount from the initial experiments. Water was added to the plate at the volumes indicated in Table 1. Next, the gradient dispensing function of the Equator™ system was used to titrate substrate by volume 1:2 across the plate using a different substrate concentration per instrument tip. Enzyme was added to the plate, followed by ATP to initiate the reactions. The kinase reactions were incubated for the predetermined reaction time, and Kinase-Glo® Plus Reagent was added to stop the reactions. Luminescence was recorded with the BMG PHERAstar plate reader. Optimal substrate concentration was defined as the lowest concentration of substrate that gave the largest dynamic range from the no-enzyme control in the linear portion of the titration curve (Figure 3, Table 3).

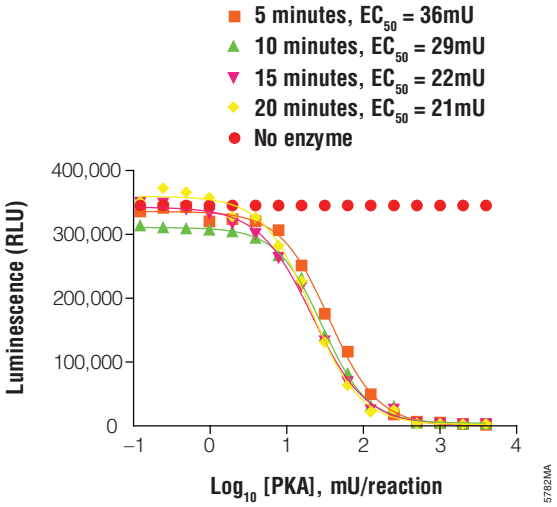

Figure 2. PKA titration results. We determined the optimal kinase reaction time for PKA to be 15 minutes. Using the curve for 15 minutes, the optimal kinase amount was determined to be 130mU per reaction. Each titration point represents an average of four replicates. Titration curves were generated using GraphPad Prism® 4.0 software.

Table 2. Enzyme Titration Results Showing Optimal Reaction Times and Corresponding Kinase Amounts.

Kinase	Reaction Time	Amount of Kinase per Reaction
PKA	15 minutes	130mU
Src	60 minutes	30mU
MAPKAP Kinase 2	90 minutes	75mU
PI3 Kinase	120 minutes	50ng

Assessing Assay Quality by Z´-Factor Analysis

A Z′-factor test is performed to assess the suitability of the optimized kinase assays for subsequent profiling applications (Table 4). Z′-factor values are statistical measures of assay reproducibility and variability (2). The volumes used for the Z´-factor analysis are provided in Table 1.

To perform the Z´-factor analysis, water was added to the assay plate. During the profiling experiments, the water was replaced with test compound. Next, a combination of enzyme and substrate at predetermined concentrations was added to the plate, followed by ATP to initiate the reaction. Reactions were incubated according to the predetermined reaction times, and then Kinase-Glo® Plus Reagent was added to each well to stop the reactions. Luminescence was recorded with the BMG PHERAstar plate reader.

Kinase Profiling for Selectivity and Potency of Test Compounds

Kinase profiling for selectivity and potency can help determine specificity and strength of inhibition of test compounds for their target kinases. After we determined optimal kinase reaction conditions and assessed Z′-factor values, we screened the kinase panel with ten compounds

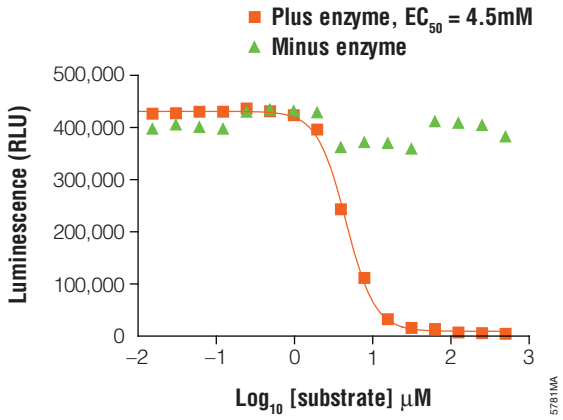

Figure 3. Kemptide titration results. Optimal reaction time and kinase amount were used to determine optimal substrate concentration. For PKA, optimal kemptide substrate concentration was determined to be 30µm kemptide. Each titration point represents an average of four replicates. Titration curves were generated using GraphPad Prism® 4.0 software.

Table 3. Substrate Titration Results Showing Optimal Substrate.

Kinase	Substrate
PKA	30µM kemptide
Src	40µM peptide 2
MAPKAP Kinase 2	40µM MAPKAP substrate
PI3 Kinase	8µg LA-phosphatidylinositol

Table 4. Z´-Factor Analysis.

Kinase	Z´-Factor
PKA	0.84
Src	0.78
MAPKAP Kinase 2	0.85
PI3 Kinase	0.84

All of the optimized kinase assays produced Z´-factor above 0.7 and were considered acceptable for kinase profiling.

of interest. The compound panel used for this application was comprised of a number of known kinase inhibitors for each respective panel kinase. The purpose of the experiment was to generate dose response curves for each test compound with each kinase. The resulting IC_{50} data were used to generate an inhibitory profile for each kinase and test compound combination.

To perform the profiling screen, five percent DMSO was added to the assay plate at varying volumes as indicated in Table 1. This DMSO backfill assured that each well contained the same final DMSO percentage following the test compound titration. Next, the gradient dispensing function of the Equator™ system was used to titrate compounds by volume 1:2 across the plate using a different compound concentration per instrument tip. A combined mixture of kinase enzyme and substrate was

Table 5. Results from 24-Point Dose Response Profile.

	PKA	Src	MAPKAP K2	PI3K
staurosporine	23nM	22nM	9µM	>100µM
PKI	27nM	>100µM	no effect	no effect
H89	186nM	510nM	100µM	no effect
rottlerin	1µM	3µM	14µM	>100µM
wortmannin	37µM	>100µM	no effect	14nM
LY 294002	19µM	no effect	no effect	9µM
quercetin	>100µM	13µM	no effect	no effect
SU 6656	>100µM	179nM	no effect	no effect
PP2	>100µM	331nM	no effect	>100µM
Src inhibitor #1	>100µM	196nM	no effect	no effect

Results represent compound IC_{50} values unless otherwise noted. Compounds that inhibit kinase activity with an IC_{50} of 1µM or less are highlighted in red. Compounds that inhibit kinase activity with an IC_{50} greater than 1µM, or had no effect on enzyme activity, are highlighted in orange. GraphPad Prism® 4.0 software was used to generate the titration curves and IC_{50} values.

added to the well, followed by ATP to initiate the reactions. The final concentration of each test compound ranged from 0.01nM to 100µM for the 24-point dose response curve, with a final DMSO concentration of 1% per reaction. Kinase-Glo® Plus Reagent was added after incubation (optimal times as previously determined above), and luminescence was recorded with the BMG PHERAstar plate reader.

Profile data are arranged in table format to show side-by-side results from the various enzyme/test compound combinations (Table 5). Test compounds that inhibit enzyme activity within the dose range tested are recorded as an IC_{50}. Test compounds that show weak inhibition at higher doses are recorded as having an IC_{50} greater than the highest concentration tested (100µM). For these compounds, an accurate IC_{50} could not be determined because maximum inhibition at higher doses did not occur. Test compounds that show no effect on enzyme activity are recorded as such. Here, data is color coded for easy visualization and to better highlight compounds that fall within limits of acceptability for potency. For this study, 1µM is chosen as a potency cutoff for compounds of interest. Compounds that exhibit a potency of 1µM or less, in addition to being selective, would be considered for further lead optimization. Green represents test compounds that inhibit kinase activity with an IC_{50} of 1µM or less. Red represents test compounds that inhibit kinase activity with an IC_{50} greater than 1µM or have no effect on enzyme activity.

Results show that staurosporine is not selective because it inhibits several kinases. In addition, staurosporine shows potent inhibition of both PKA and Src enzymes. H89 is also nonselective and shows potency for both PKA and Src. However PKI (PKA), rottlerin (PKA), wortmannin (PI3 kinase), SU 6656 (Src), PP2 (Src), and Src inhibitor #1 (Src) showed both potency and selectivity. These compounds would be of greatest interest for lead optimization as inhibitors for their respective kinase enzyme drug targets.

Summary

Kinase profiling is an important component of the drug discovery process. Profiling with IC_{50} provides simultaneous potency and selectivity information about compounds of interest. Profiling data assist in the detection of inhibitors that may have potential toxic effects due to off-target activity. Compounds that exhibit the strongest potency and specificity profiles can be identified as leads for further optimization. Miniaturizing this profiling process in uHTS format generates data from a wide range of compound concentrations, providing more meaningful and accurate information. The combination of the Kinase-Glo® Plus Assay with low-volume liquid dispensing equipment provides an excellent solution for assay optimization and kinase profiling.

References

1. Cohen, P. (2002) *Nat. Rev.: Drug Disc.* **1**, 309 –15.
2. Zhang, J. *et al.* (1999) *J. Biomol. Screen.* **4**, 67–73.

Protocols

- *Kinase-Glo® Plus Luminescent Kinase Assay Technical Bulletin* #TB343, Promega Corporation.
  www.promega.com/tbs/tb343/tb343.html

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.