A Flexible Workflow for Automated Bioluminescent Kinase Selectivity Profiling

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Abstract
Kinase profiling during drug discovery is a necessary process to confirm inhibitor selectivity and assess off-target activities. However, cost and logistical limitations prevent profiling activities from being performed in-house. We describe the development of an automated and flexible kinase profiling workflow that combines ready-to-use kinase enzymes and substrates in convenient eight-tube strips, a bench-top liquid handling device, ADP-Glo Kinase Assay (Promega, Madison, WI) technology to quantify enzyme activity, and a multimode detection instrument. Automated methods were developed for kinase reactions and quantification reactions to be assembled on a Gilson (Middleton, WI) PIPETMAX, following standardized plate layouts for single- and multidose compound profiling. Pipetting protocols were customized at runtime based on user-provided information, including compound number, increment for compound titrations, and number of kinase families to use. After the automated liquid handling procedures, a GloMax Discover (Promega) microplate reader preloaded with SMART protocols was used for luminescence detection and automatic data analysis. The functionality of the automated workflow was evaluated with several compound-kinase combinations in single-dose or dose-response profiling formats. Known target-specific inhibitions were confirmed. Novel small molecule-kinase interactions, including off-target inhibitions, were identified and confirmed in secondary studies. By adopting this streamlined profiling process, researchers can quickly and efficiently profile compounds of interest on site.

Keywords
kinase profiling, bioluminescence, ADP detection, selectivity profiles, liquid handling

Introduction
Kinases are a large family (>500 members) of phosphotransferases that regulate a diverse set of biological processes such as cellular growth, division, and differentiation.1,2 Disruption of these biological processes due to aberrant kinase enzyme activity leads to a multitude of diseases such as cancer, inflammation, and diabetes. As a result, kinases have been one of the most targeted enzyme classes in several therapeutic research areas,3,4 with about 30 kinase-based drugs approved by the Food and Drug Administration (FDA). Currently, many drug discovery programs are devoted to the identification of more kinase inhibitors with diverse modes of action.4,5 Achieving the right balance between potency and selectivity of kinase drugs remains a major challenge.6 One reason is that most small molecules target the evolutionarily conserved adenosine triphosphate (ATP) binding pocket present in all kinases. Therefore, it is arduous to identify therapeutic compounds that will inhibit the kinase target with high selectivity and yet not cause side effects by affecting other kinases involved in crucial signaling pathways. To better understand the mode of action of lead compounds and avoid potential toxicities in the clinic,7 small-molecule candidates are profiled early in the drug discovery process against various liability panels, including protein kinases.

Numerous technologies that assess kinase activity have been developed and used successfully to map small molecule–kinase interactions in vitro.8,9 Traditionally, these technologies are used to measure the effect of small molecules on the target kinases in high throughput or smaller scale mode-of-action study settings. For profiling, these technologies have typically been offered by service providers in a fee-for-service model.10,11 To facilitate in-house kinase profiling, we reported on the

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development of accessible standardized profiling systems for 112 kinases covering all branches of the kinome. These systems contain sets of multitube strips comprising eight kinase enzymes that have been standardized for consistent kinase activity using the well-established bioluminescent ADP-Glo (Promega, Madison, WI) kinase assay. We showed that by using this system, we could create diverse selectivity profiles for small-molecule inhibitors using small or large kinase panels. The streamlined protocol developed for the kinase profiling strips can be performed in either a manual or automated format. The protocol is easy to perform and requires only one simple dilution of the kinase and substrate strips before dispensing into assay plates. Although profiling data can be generated manually for kinase panels using the strips, diluting compounds and dispensing kinases can be time-consuming and challenging to set up. When evaluating larger numbers of kinases, it may be preferable to adopt the use of automation and liquid handling instruments to enhance the profiling workflow. However, automation can be daunting for many users as it requires both the selection of an appropriate liquid handling instrument and creation of automated methods to execute the successive liquid dispensing steps required for the multiple kinase reactions assembly.

We present the development of an automated and flexible kinase profiling workflow that encompasses kinase reaction assembly, bioluminescence detection, and data analysis that are automatically conducted according to user-input parameters for single-dose or dose-response kinase inhibitor profiling. By incorporating a simple and affordable bench-top liquid handling device (PIPETMAX; Gilson, Middleton, WI) and detection instrument (GloMax Discover; Promega) to the kinase strips concept, we have created a streamlined workflow for kinase profiling amenable to even the novice automation user.

Materials and Methods

Reagents

Kinase inhibitor compounds were purchased from the following companies: bosutinib, imatinib, PF-477736, ponatinib, tofacitinib, VX-702, and staurosporine from LC Laboratories (Woburn, MA) and kenpaullone from Tocris (Baldwin, MO). All compounds were prepared as 1-mM stocks in DMSO and stored at −20 °C until use.

Kinase Selectivity Profiling Systems (KSPS) (General Panel [V6928], CMGC-1 [V6854], TK-2 [V6852], TK-4 [V6922], CAMK-1 [V9632], STE-1 [V6916]) and Kinase Enzyme Systems (KES) (JAK3 [V3701]) were obtained from Promega and stored at −70 °C until use. KSPS and KES kits included kinases, substrates, 5× Reaction Buffer A, and 0.1 M dithiothreitol (DTT). The kinases included in each KSPS used in this study are shown in Figure 1A.

ADP-Glo Kinase Assay, including 10 mM Ultra-pure ATP (V9102; Promega), was stored at −20 °C until use. ADP-Glo Kinase Assay consists of two reagents: ADP-Glo Reagent and Kinase Detection Reagent. Kinase Detection Reagent was prepared according to the manufacturer’s instructions by combining Kinase Detection Buffer with Kinase Detection Substrate.

Nuclease-free water (P1195; Promega) was used to dilute 5× Reaction Buffer A and Ultra-pure ATP. Buffers and reagents were manually prepared prior to automated reaction assembly. Kinase Buffer at 4× concentration was made by diluting 5× Reaction Buffer A with nuclease-free water and supplementing with 200 µM DTT. Kinase Buffer at 4× was further diluted with water to 2.5× concentration to be used for automated kinase dilution. Compound diluent was created by diluting 4× Kinase Buffer to 1× with nuclease-free water and supplementing with 5% DMSO. Then, 80 µM ATP was created using nuclease-free water and later used for automated preparation of the substrate strips.

Working stocks of test compounds were made by diluting the 1-mM stocks with 4× Kinase Buffer and water to achieve a 5× compound concentration of 50 µM in 1× Kinase Buffer.

Plastic Consumables

Opaque white low-volume 384-well polystyrene assay plates (4512; Corning, Corning, NY) were used in this study. Clear V-bottom 96-well plates (3897; Corning) served as compound dilution plates and reagent source plates on the PIPETMAX. The 20-µL tips (DSL10ST F172211; Gilson) and 200-µL tips (DS200ST F172311; Gilson) were used in all automated liquid handling procedures.

Instrumentation and Accessories

Automated liquid handling was carried out using a Gilson PIPETMAX, a robotic platform equipped with two motorized eight-channel air displacement pipettes. The system included a PIPETMAX 268 with standard cover (32100000; Gilson), a MAX8x20 pipette head (FC10022) for volumes of 1 to 20 µL, a MAX8x200 pipette head (FC10022) for volumes of 20 to 200 µL, and PIPETMAX 268 Tray (32000091) with a capacity of up to nine bed elements that meet the SLAS standards for microplate footprints. A 96-well passive cooling block (496PT Rack; Gilson) was used for KSPS enzymes and substrates. The PIPETMAX instrument was controlled with an external PC running TRILUTION micro software (32000320; Gilson).

A GloMax Discover System (GM3000; Promega) with SMART protocols for single-dose inhibition and inhibitor dose-response was used for luminescence detection and data analysis. SMART protocols read the plate and perform data analysis according to the KSPS experimental setup.
Experimental Procedures

Two PIPETMAX protocols were developed for this study: one protocol for conducting experiments in single-dose screening mode using the KSPS General Panel (24 kinases), the other protocol for performing multipoint dose-response experiments using KSPS families of kinases. A single-dose screen of the General Panel was first performed at a 1-µM concentration of each inhibitor. Inhibitors exhibiting both on- and off-target effects were further evaluated in a 10-point dose-response secondary screen using selected KSPS families of enzymes.
Variables set within each protocol guided the assay setup according to the number of compounds and kinase families being profiled. The dilution series of compound, if applicable, was user-defined and adjusted on-the-fly within the automated method. The plate layout used for either single-dose or dose-response profiling was standardized according to the KSPS assay technical manual and assembled in a manner compatible with the GloMax Discover SMART protocols. For detailed instructions on reagent preparation and automation setup, please see the setup guide entitled *Automation of Kinase Selectivity Profiling System with PIPETMAX*.18

**Single-Dose Profile**

The 496PT cooling block was held at −20 °C prior to beginning the experiment. The KSPS General Panel and substrate strips were thawed on ice. Working buffers, ATP solution, test compounds, and ADP-Glo reagents were prepared as previously described and arrayed to the wells of a Corning 3897 source plate that was placed on the deck of the PIPETMAX. The cooling block containing kinase and substrate strips was then placed onto the bed of the PIPETMAX just prior to running the automated protocol.

The single-dose profile protocol began by diluting the kinase and substrate strips with 95 µL of 2.5× Kinase Buffer and 20 µL of 80 µM ATP solutions, respectively (Fig. 1B). One microliter of test compound (5×) was arrayed to the assay plate in duplicate wells, followed by the addition of 2 µL kinase. No-compound (maximum kinase activity) and no-kinase (background) controls were also assembled for every kinase. The assay plate was manually transferred to a centrifuge for a brief spin at 1000 rpm, then placed back on the instrument bed for a 10-min room temperature incubation. Two microliters of ATP/substrate working solution was added to the reactions, followed by another brief plate centrifugation at 1000 rpm and a 60-min room temperature incubation. The kinase reaction was terminated by adding 5 µL ADP-Glo Reagent to the plate followed by plate centrifugation at 1000 rpm and a 40-min room temperature incubation, which depletes all remaining ATP. Last, 10 µL of Kinase Detection Reagent was dispensed to the plate, followed by plate centrifugation at 1000 rpm and a 30-min room temperature incubation to convert all adenosine diphosphate (ADP) to ATP and then ATP to light in a luciferase/luciferin reaction. The GloMax Discover SMART Protocol for single-dose inhibition was then used for luminescence quantification and subsequent off-line analysis of % inhibition (Fig. 2).

**Dose-Response Profile**

Cooling block preparation, reagent preparation, and array to the source plate occurred as previously described for the single-dose profile. The dose-response profile protocol began by diluting the kinase and substrate strips with 2.5× Kinase Buffer and ATP solutions, respectively. A 10-point serial 1:4 titration of test compound was then performed prior to compound array to the assay plate. The kinase reaction assembly procedure occurred in the same manner as described above for the single-dose profile (Figs. 1B and 2). The GloMax Discover SMART Protocol for inhibitor dose-response was then used for luminescence quantification and subsequent off-line determination of IC\textsubscript{50}.

**Data Analysis**

In every experiment, no-enzyme and no-compound control reactions were included to represent background luminescence (0% activity) and uninhibited kinase activity (100% activity), respectively. Using Microsoft Excel (Microsoft, Redmond, WA), the SMART protocols calculated and reported percent kinase activity by subtracting the average no-enzyme control luminescence values from all kinase-containing reactions with or without compound, then converting these net luminescence values to percent activity based on the no-compound control reactions representing 100% kinase activity. The SMART protocol for inhibitor dose-response included additional mathematical calculations for IC\textsubscript{50} using a published method.19

**Results and Discussion**

To streamline kinase inhibitor profiling, we previously created accessible standardized profiling systems for a large number of protein kinases covering all branches of the kinaseome.12 These systems consist of different sets of kinases and their corresponding substrates ready to use in multtube strips. The kinase stocks are standardized for optimal kinase activity, which is detected with a bioluminescent ADP detection assay. We showed that these strips can routinely generate inhibitor selectivity profiles for focused or broad kinase family panels. Although this manual approach yielded accurate profiling data,13 it would be more efficient and less time-consuming if this approach was automated in order to facilitate routine in-house profiling.

A variety of automated liquid handlers are capable of accurately titrating and dispensing compounds, as well as delivering biochemical reaction components and assay reagents to the desired plate format. Screening instruments, such as bulk dispensers, are affordable and well adapted to the delivery of assay components for testing a single target with large compound libraries, but their use may prove to be challenging when used in a smaller scale kinase profiling mode when different kinase/substrate combinations need to be added to the same assay plate. Serial titration and small volume delivery of compound can be achieved through the use of noncontact instrumentation such as an acoustic
dispenser, but the cost of this type of instrumentation is often out of reach for the low-throughput user looking to bring profiling projects in-house. For smaller scale, in-house profiling activities, it is therefore more economical to have an automated liquid handler with the flexibility to aspirate and dispense multiple reagent types, titrate compounds, and accurately deliver small volumes of reaction components to an assay plate. We chose the PIPETMAX from Gilson to develop an automated kinase profiling workflow because of its affordability, small footprint, and inclusion of the aforementioned features for assay assembly. We created an automated and flexible kinase profiling workflow that includes kinase reaction assembly and assay reagent dispensing with the PIPETMAX, followed by kinase activity signal detection and data analysis with the multimode detection instrument, GloMax Discover (Fig. 2A). This workflow is orchestrated by automation protocols involving simple user-input parameters (Fig. 2B) that guide assay assembly for either single-dose or dose-response kinase inhibitor profiling experiments.
Evaluation of the Dose-Response Automated Kinase Profiling Process

The PIPETMAX automation protocols were developed in accordance with the KSPS assay plate setup requirements as outlined in the product technical manual. We tested the automated dose-response profiling workflow using the CAMK-1 KSPS as a model for inhibition by PF-477736 compound, a CHK1/2 inhibitor. Using the same preparation of reagents, dose-response testing was performed simultaneously using automated or manual reaction assembly. As shown in Figure 3, IC_{50} values and inhibition rank order were in agreement between the manual method and the automated process performed with the PIPETMAX. These results suggested that the automated program accurately titrated the compound and assembled the reactions in a manner that was consistent with the manual dispensing skills of a trained scientist familiar with the assay procedure. As the liquid handling tasks for both the single-dose and dose-response protocols are very similar, the comparative results obtained from this automated versus manual dose-response experiment indicated that the single-dose automated protocol was ready for testing with the General Panel of kinases.

Automated Single-Dose Screening of the General Panel

In a typical kinase inhibitor discovery campaign, chemical compounds identified as inhibitors of the targeted kinase are profiled against a customized panel of kinases to assess their selectivity. To test our automation workflow with a larger panel of kinases and chemical compounds, a general panel of 24 kinases from different branches of the kinome (Fig. 1A) was chosen for selectivity assessment of seven known small-molecule kinase inhibitors. Three types of compounds were used in this study: (1) compounds that inhibit specific kinases in the panel such as tofacitinib (JAK3 inhibitor), VX-702 (p38α inhibitor), and kenpaullone (GSK3β inhibitor); (2) compounds that are known to inhibit multiple kinases in addition to the target kinase, such as ponatinib (targets ABL but also inhibits FGFR) and bosutinib (targets ABL but also inhibits the Src family of kinases); and (3) control compounds that will either affect many kinases in the panel, such as the pan kinase inhibitor staurosporine, or not affect any kinase in the general panel such as the ABL kinase inhibitor imatinib. The single-dose profiling protocol described in the Materials and Methods was used for the experiment. Data were processed through the SMART protocol present in the GloMax Discover software to generate a three-color heatmap representing the percent activity remaining for each kinase in the presence of compound (Fig. 4). The inhibition profile was consistent with previously published literature suggesting that the automated single-dose kinase profiling protocol could be used for accurate detection of known inhibitor activities. As predicted, tofacitinib, kenpaullone, and VX-702 strongly inhibited their respective targets, JAK3, GSK3β, and p38α, respectively. The pan–kinase inhibitor staurosporine inhibited almost all of the kinases in the panel while imatinib showed no kinase inhibition. Although the secondary targets LCK and SYK for bosutinib and FGFR1 for ponatinib were inhibited similarly to what was reported, these two multikinase-inhibiting compounds generated previously undocumented off-target inhibition; bosutinib and ponatinib inhibited with high potency MINK1 and p38α MAPK, respectively.
Confirmation of Kinase Inhibition Using the Automated Dose-Response Profile Protocol

Single-dose profiles generally provide an indication of the effect of compounds on selected kinases. To confirm inhibition results obtained in our single-dose profiling experiment and qualify the automated dose-response profile protocol, we performed dose-responses of the inhibitors against selected KSPS families containing the primary and secondary target kinases for each of the compounds. The purpose of using KSPS family strips in this experiment has the additional advantage of identifying the effect of each compound on homologous kinases or kinases belonging to the same subfamily as the target.

Figure 5A,D shows that VX-702 inhibited the target kinase p38α and its closely related kinase p38β with high potency, but none of the other p38 kinases or CMGC kinases were inhibited. Because we used the CMGC kinase family strip instead of just single kinases for the confirmation, we could identify the rank order of inhibitions against the p38 MAPK family and the other kinases in this kinome branch. Similarly, using the dose-response protocol, we confirmed the inhibitions of ponatinib against the target ABL1 kinase and known secondary targets (Fig. 5B–D) with IC50 values comparable to reported values. Data were collected using the single-dose SMART protocol, which calculates the percent kinase activity by subtracting the no-enzyme control luminescence from all kinase-containing reactions and then converting the net luminescence values to percent activity based on the no-compound control reactions. Percentages of activity remaining for each kinase/compound pair are shown as a heatmap representing different kinase activity ranges.
Investigating Inhibitor Off-Target Activities

In Figure 4, we showed that ponatinib and bosutinib generated previously unreported kinase inhibitions against p38α and MINK1 kinases, respectively. We wanted to confirm these off-target inhibitions and define their extent by generating an IC_{50} using our automated dose-response profiling protocol. Because of the ease of use and flexibility of the kinase selectivity profiling systems, we used whole family strips to generate inhibitor IC_{50} values for the off-target inhibition and identify any additional effect on closely related kinases. Using the CMGC-1 kinase family strip, ponatinib was confirmed to inhibit p38α but also was found to inhibit the closely related kinase p38β within a similar IC_{50} range (Fig. 6A). No other kinase in this family was inhibited by ponatinib, suggesting that the mechanism of this off-target inhibition is specific to two closely related p38 MAPKs. Moreover, to confirm that the observed off-target activity is not due to an artifact of the technology used, we performed the same dose-response and used an orthogonal assay that detects the ATP depletion after a kinase reaction instead of ADP detection. We found that ponatinib inhibits the p38α kinase with similar IC_{50} while it does not have any effect on the reagent’s performance (Suppl. Fig. S1A). It should be noted that ponatinib had an off-target activity against JAK3 as well in the single-dose General Panel profile experiment (Fig. 4). We confirmed that this inhibition is real, and the 40-nM IC_{50} value that was obtained was in the same range as previously reported (data not shown).24 Similarly, using the STE-1 family strip or a single MINK1 kinase, we confirmed the off-target inhibition of the serine/threonine kinase MINK1 by bosutinib in the ADP detection and ATP depletion assays (Fig. 6B and Suppl. Fig. S1B).

Surprisingly, the multi–tyrosine kinase inhibitor bosutinib not only inhibited MINK1 but also inhibited many of the other serine/threonine kinases belonging to the same STE kinome family included in the STE-1 strip. Bosutinib inhibited TNIK and HPK1 kinases with even higher potency than MINK1 (IC_{50}s: 3, 4, and 29 nM, respectively). These findings suggested that inhibitor off-target activities identified during single-dose profiling can be expanded to kinases closely related to or belonging to the same kinome branch of the off-target kinase. Using multiple related kinases when confirming off-target activities...
proved here to be useful in understanding the extent of inhibitor promiscuity.

**Reproducibility of the Automated Liquid Dispensing**

Performing the kinase selectivity profiling using automated reagent dispensing with PIPETMAX produced inhibition profiles similar to previous literature reports. We wanted to confirm the reproducibility of data generated between different PIPETMAX instruments. We repeated the bosutinib dose-response against the STE-1 kinase strip described in Figure 6B to evaluate interplatform capabilities for IC_{50} generation. Kinase reactions were assembled on two independent PIPETMAX instruments using identical experimental parameters. As shown in Figure 6C, MINK1 inhibition curves and the overlaid bosutinib titration results suggest that comparable inhibition data can be achieved between different instruments. Moreover, using replicate data generated from both instrument runs, we calculated the signal-to-background (S/B) ratio and assessed the variability of IC_{50} generated. The S/B and the percent coefficient of variation (CV) of the IC_{50} values generated were 74-fold and 7.4%, respectively, indicating the high sensitivity and the reproducibility of the automated assay (Fig. 6C). We have confirmed here that the PIPETMAX protocols (http://www.gilson.com/kinaseprofilingbundle) can be transferred to different instruments to yield reproducible results for KSPS profiling applications.

In conclusion, kinase profiling is a crucial step of kinase-related drug development to determine whether small-molecule inhibitors are specific to the kinase target or to what extent the compounds demonstrate any off-target activity. Kinase selectivity profiling can be performed in-house or outsourced to service providers. To assess selectivity of a compound in-house, many challenges can be encountered that are related to choice of kinase assay, kinases to be profiled, reagent dispensing mode, and data analysis software. Here we report how a ready-to-use bioluminescent KSPS, combined with easy to use automation (PIPETMAX), signal detection (GloMax Discover), and data analysis (SMART protocols), supports a streamlined workflow to enable quick and efficient in-house kinase inhibitor profiling. Our workflow relies on the standardized kinases supplied in KSPS, which require no assay development and are assayed using the luminescent ADP detection kinase assay, a universal platform that has been validated with a large number of kinases for drug discovery, enzyme characterizations, and inhibitor mode-of-action studies. Automation protocols were developed for kinase profiling to allow bench-top liquid handling for either single-dose or dose-response profiling modes. Finally, the kinase activity-related signal and the automatic data analysis were performed using integrated protocols in the GloMax Discover multimode.
detection system. Using this automated profiling workflow, single-dose inhibitions and dose-response curves were generated rapidly. Compound potencies, or IC50 values, were comparable to those previously reported with manual profiling or with other methods. The benefits of using KSPS enable the user to overcome the associated technical challenges inherent to other methods, such as lengthy assay development, safety issues due to radioactivity usage, or inconveniences related to outsourcing. Using this kinase profiling workflow, known compound off-target activities were confirmed and new activities were identified. The use of kinase family KSPS strips for compound potency confirmation provided an additional advantage, allowing the identification of compound potencies against the closely related kinases or kinases belonging to the same kinome branch of the off-target kinase. This flexible workflow for automated kinase selectivity profiling can be easily adopted by scientists with little to no automation experience for regular in-house kinase inhibitor profiling.

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Declaration of Conflicting Interests

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