Flexible Automated Workflow for Kinase Selectivity Profiling using PIPETMAX®

Application Note TRANS0216

Kinase inhibitors are an important class of therapeutics, and kinase profiling to confirm inhibitor selectivity and assess off-target activity is a critical step during the drug discovery process. However, performing these activities in the research lab is often cost-prohibitive. Further, manual pipetting into 384-well plates can be error prone and tedious. Here we demonstrate the use of pre-configured kinase enzyme profiling systems on affordable automated liquid handling and bioluminescence detection instrumentation, which enables researchers to quickly profile their compounds of interest on-site, generating reliable and reproducible data on demand and on their timeline.

Introduction

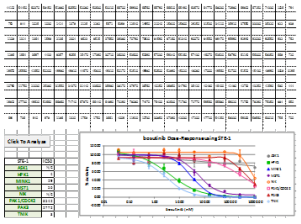
Protein kinases regulate key cellular functions including signal transduction, cell division, and apoptosis. This enzyme group constitutes one of the largest (>500) and most functionally diverse gene families and has been implicated in cancer and various diseases. Deregulation of kinases by small molecule inhibitors has become a standardized therapy treatment.[1]

Kinase profiling is a necessary part of the drug discovery process in order to confirm that small molecule inhibitors are specific to the protein kinase of interest, and to determine whether the inhibitor demonstrates off-target activity. Optimizing kinase reactions and maintaining a library of purified enzymes can be so arduous that labs choose to outsource their kinase profiling activities.

Here we report how a ready-to-use Kinase Selectivity Profiling System (KSPS) from Promega Corporation, combined with easy to use automation, signal detection, and data analysis (Figure 1) supports a streamlined workflow to enable quick and efficient in-house kinase inhibitor profiling.

PIPETMAX® Liquid Handling

GloMax® Discover Detection



SMART Protocol Data Analysis

Figure 1. Reaction assembly, detection, and data analysis are automatically conducted according to user-input parameters for single or dose-response testing of up to two kinase strips per plate.

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Materials & Methods

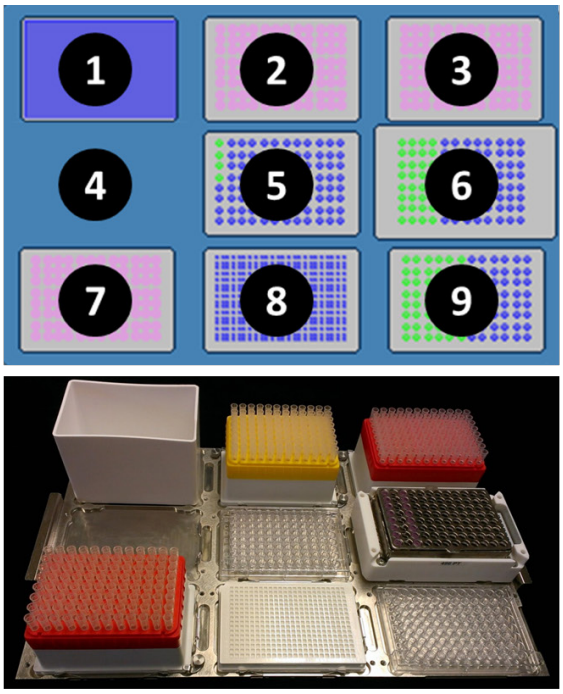
Enzymes and reagents

Commercial KSPS kits containing 8-tube strips of kinases and substrates were obtained from Promega Corp, Madison WI and used according to manufacturer's directions.[2] The ADP-Glo™ Kinase Assay[3] (V6930, Promega) was used to quantify kinase activity. Stock solutions of inhibitor compounds were diluted to 1mM in DMSO before use.

Automated reaction setup liquid handling

Kinase enzyme reaction setup, including compound dilution and preparation of kinase working stock and ATP/substrate working stock, was carried out using a PIPETMAX® automated liquid handler (Gilson, Inc., Middleton WI). Reactions contained 1µL of compound, 2µL of kinase working stock and 2µL of the corresponding ATP/substrate working stock. Addition of ADP-Glo and kinase detection reagent was also carried out using PIPETMAX. Negative controls (no compound and no compound/no kinase) were included on each plate.

Automated KSPS protocols for PIPETMAX (www.gilson.com) were developed and tested by Promega. Protocols were imported into TRILUTION micro 2.0 software (Gilson). Kinase and substrate strip tubes were placed in a cold (~0°C) passive thermal block with metal overlay (Rack 496 PT, Gilson, Inc.). Compound serial dilution was performed in a Costar 3897 V-bottom 96 well plate. Reagents (compound diluent, ATP, 2.5x Kinase Buffer, ADP-Glo Reagent and Kinase Detection Reagent) were placed in a Costar 3897 V-bottom 96 well plate as directed by the protocol. Kinase assay reactions were assembled in a white low volume Corning 4512 384-well assay plate (Figure 2).



1. Tip waste
2. DS200ST tips in tip adapter block
3. DSL10ST tips in tip adapter block
4. Empty
5. Test compounds in positions A1-D1 of 96 well V-bottom plate, Corning 3897.
6. Strip tubes containing kinase enzyme (columns 1 and 2) and substrate (columns 3 and 4) in passive thermal block 496PT (chilled to 0°C)
7. DSL10ST tips in tip adapter block
8. 384 well assay plate, white, Corning 4512.
9. Assay reagents in positions A1-H6 of 96 well V-bottom plate, Corning 3897.

Figure 2. Schematic and photograph of PIPETMAX bed layout for automated Kinase Selectivity Profiling Systems. Liquid handling steps are performed by PIPETMAX, using the bed layout and labware indicated. Kinases and substrates /cofactor stocks are each provided in an 8-tube strip and placed in a cold block. One-step dilutions directly in these strips produce sufficient Kinase and ATP/Substrate Working Stocks for 25 kinase reactions in a 384-well plate. After 1-hour incubation in the presence of inhibitor, kinase activity is quantified using the ADP-Glo Kinase Assay. The resulting luminescent signal is proportional to kinase activity.

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Liquid handling was carried out on PIPETMAX with tips validated for use with 384-well plates (DS200ST and DSL10ST). Centrifugation, mixing, and detection were performed off-bed, while room temperature reaction incubations were performed either directly on the bed of PIPETMAX® instrument or on an off-bed temperature-controlled heat block held at 25°C.

Detection and data analysis

After the final incubation, the assay plate was transferred to a GloMax® Discover plate reader (Promega) and luminescence data were collected via the Kinase Selectivity Profiling SMART Protocol. The Solver and Analyze functions within Microsoft® Excel® Macro-Enabled worksheets for the Kinase Selectivity Profiling System Inhibitor Dose Response and Single Dose Inhibition protocols were used for automated data analysis to graph the data, fit curves, and calculate IC_{50} values.[4]

TK-2	TK-4	CMGC-1	STE-1	General Panel		
ABL1	c-MER	ERK2	ASK1	FGFR1	CDK2/CyclinE1	AKT1
BRK	FGFR1	GSK3b	HPK1	JAK3	GSK3b	PKCa
BTK	FGFR2	JNK1	MINK1	LCK	p38a	ROCK1
CSK	FGFR4	JNK3	MST1	SYK	AMPK A1/B1/G2	Aurora A
FYN A	FLT1	p38a	NIK	MINK1	CAMK4	CK2a1
LCK	FMS	p38b	PAK1/CDC42	PAK1/CDC42	CHK1	IKKb
LYN B	MET	p38d	PAK3	IRAK4	DAPK1	CK1a1
SRC	RET	p38g	TNIK	TAK1-TAB1	MAPKAPK2	CK1g1

Figure 3. The Promega Kinase Selectivity Profiling Systems contain 8 kinase and substrate pairs that are grouped by family (e.g. TK, CMGC, and STE) or as a General Panel. The color coding corresponds to different kinase families. 17 different KSPS kinase strips are available in the Promega catalog, and custom combinations of enzymes can also be ordered.

Results and Discussion

Screening of selective and off-target kinase inhibitor activity was easily streamlined in a general laboratory setting using pre-configured kinase reactions (KSPS) and automated liquid handling, detection, and analysis programs. Several products are available in this line; kinases are grouped either in single family strips or as a general panel representative of the human kinome for a broad kinase profile (Figure 3).

For these experiments, the Gilson PIPETMAX was used to perform all liquid handling steps. The user selects either the single-dose or dose-response protocol and enters in the variables within the TRILUTION® micro run software to define the volume ratios for compound serial dilution (if applicable), dictate the number of kinase and substrate strips to prepare for the run, and guide reaction setup in the assay plate. Reactions are prepared according to templates that are pre-configured for the multimode plate reader (GloMax, Promega Corporation) and Smart Protocol data analysis files.

A single point selectivity screen was performed with nine test compounds (1µM) and 24 unique kinases in one 384-well plate using automation and three KSPS general kinase panels. As shown in Table 1, staurosporine ubiquitously inhibited several kinases, whereas VX-702 and tofacitinib were selective in inhibiting p38α and JAK3, respectively. Ponatinib shows expected on-target inhibition of the Abl-associated LCK and FGFR tyrosine kinases, but unexpected off-target inhibition of p38α. Subsequent dose-response experiments with ponatinib verified potency for tyrosine kinases

		Bosutinib	Imatinib	Ponatinib	Sunitinib	Tofacitinib	SU6656	VX-702	Kenpaullone	Staurosporine
GP-1	FGFR1	39	82	4	22	71	29	81	77	2
	JAK3	58	98	7	68	0	82	95	95	-1
	LCK	0	69	0	33	99	57	107	75	1
	SYK	15	85	97	55	87	60	89	39	-2
	MINK1	4	101	65	42	109	100	85	43	1
	PAK1/CDC42	69	103	125	104	103	108	92	98	32
	IRAK4	42	95	99	28	106	54	106	97	2
	TAK1-TAB1	90	102	35	30	109	101	108	89	5
GP-2	CDK2/CyclinE1	94	101	86	93	93	98	95	38	2
	GSK3β	86	90	88	74	86	88	86	5	2
	p38α	48	92	1	95	80	95	0	97	60
	AMPK A1/B1/G2	53	103	82	7	77	21	88	82	1
	CAMK4	80	101	96	25	94	101	93	96	3
	CHK1	75	109	105	26	94	102	92	94	0
	DAPK1	102	102	101	73	100	105	98	101	4
	MAPKAPK2	85	87	89	84	87	85	88	85	26
GP-3	AKT1	99	99	93	88	103	97	101	100	0
	PKCα	78	102	94	85	62	92	78	101	1
	ROCK1	68	96	93	58	95	78	87	50	1
	Aurora A	100	103	105	89	82	40	107	101	4
	CK2α1	98	97	100	94	103	103	102	85	87
	IKKβ	94	95	60	89	86	88	83	96	27
	CK1α1	84	96	99	60	89	97	88	98	81
	CK1γ1	101	94	94	50	93	97	94	98	88

Table 1. Single-dose inhibitor profile (9 compounds) against three KSPS general panels (24 total kinases). The amount of enzyme activity remaining (%) following treatment with 1µM compound is reported. Boxes highlighted in red represent >80% inhibition.

from KSPS: TK-2 and TK-4 and confirmed off-target effects with KSPS: CMGC-1 kinases (data not shown).

Likewise, bosutinib, an Src family kinase inhibitor, shows expected on-target inhibition of the LCK tyrosine kinase, but unexpected off-target inhibition of MINK1.[5] The specificity of bosutinib was further investigated in a dose-response profiling experiment with MINK1 and other like family kinases. PIPETMAX® performed a 10-point serial 1:4 titration of bosutinib, added the diluted KSPS reagents: STE-1 kinases/substrates and dispensed ATP to start the kinase reaction. After 60 min at room temperature, the ADP-Glo™ reagents were added to measure the % activity. The results showed potent inhibition against TNIK (IC_{50} = 3nM), HPK (IC_{50} = 4nM), MINK1 (IC_{50} = 29nM), MST1

(IC_{50} = 56nM), and to a lesser extent PAK/CDC42, PAK3 and NIK (Figure 4).

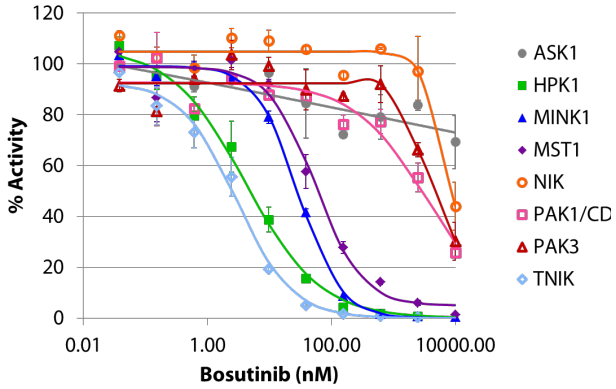

Figure 4. Dose response experiments were performed to evaluate bosutinib potency against the kinases in the STE-1 family. Reactions were performed in duplicate.

To assess reproducibility of the assay, kinase reactions were assembled on two independent PIPETMAX® instruments using the same experimental parameters. Serial dilution of bosutinib, preparation of kinase and substrate/ATP working stocks, assembly of kinase activity reactions, and dispensing of ADP-Glo™ and kinase detection reagent were carried out using a Gilson PIPETMAX automated liquid handler. As shown in Figure 5, this dose-response experiment with bosutinib and STE-1 kinase strip, with a dilution factor of 4, yielded very reproducible inhibition data. An example of MINK1 from the STE-1 KSPS is shown in Figure 5.

"…reactions assembled on two independent PIPETMAX instruments… yielded very reproducible inhibition data.

The Kinase Selectivity Profiling Systems come ready-to-use and no assay optimization is required. Complete automation, from compound dilution and addition through detection reagent dispensing, was easily and reliably achieved with PIPETMAX. These pre-configured kinase assays, coupled with automated pipetting, detection, and analysis methods, provide a streamlined workflow for convenient, in-house, single-point and dose-response kinase profiling.

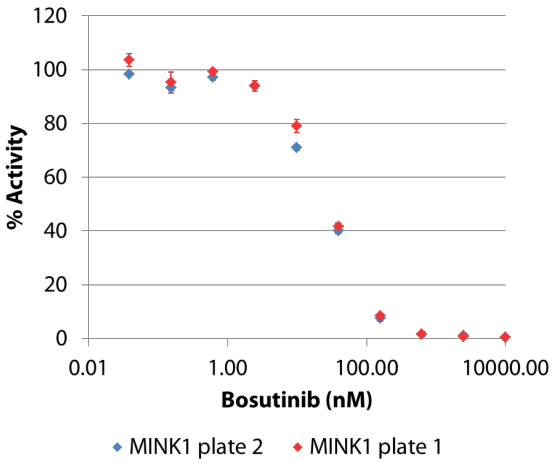

Figure 5. Reproducibility of MINK1 inhibition profile. The graph shows data from duplicate reactions assembled on two independent PIPETMAX liquid handlers (i.e. four reactions per condition).

References

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Summary

- Kinase inhibitors represent an important focus for potential cancer therapies.
- This application note demonstrates a ready to use, validated system for the systematic profiling of kinase inhibitors.
- PIPETMAX automates the assay setup of the Promega Kinase Selectivity Profiling System for both single point screening and dose response evaluation.
- The GloMax® Discover plate reader simplifies data collection and analysis with the Kinase Selectivity Profiling SMART Protocols.

Ordering Information

Item	Product Number
PIPETMAX 268 with Standard Cover	32100000
MAX8x20 Pipette head	FC10022
MAX8x200 Pipette head	FC10021
PIPETMAX 268 Tray 384-well	32000091
Tip Adapter Block (qty 3)	32000175
DSL10ST pipette tips	F172211
DS200ST pipette tips	F172311
Rack 496PT freezer block	32000238
Tip Storage Riser	32000177