A Simple and Robust Automated Kinase Profiling Platform using Luminescent ADP Accumulation Technology

Brad Larson[1], Peter Banks[1], Hicham Zegzouti[2], Said Goueli[2]

[1]BioTek Instruments, Inc. • P.O. Box 998, Highland Park • Winooski, VT 05404 • USA • [2]Promega Corporation • 2800 Woods Hollow Road • Madison, WI 53711 • USA

Overview

Kinases continue to be an important target class in today's drug discovery efforts. Following the identification of lead compounds through screening efforts, it is important to profile these leads against other kinases to ascertain potential off-target effects. Because different kinases included in a profile require the use of different buffers, substrates, ATP, and incubation times, the proper choice of assay and instrumentation is essential to minimize optimization time and cost. Here we demonstrate the versatility of a luminescent ADP accumulation assay, where one set of reagents is used for kinases with differing $ATP_{Km\ app}$. We have used a simple, inexpensive automated pipetting system to automate the entire process in 384-well format, from assay optimization through generation of compound IC_{50} values. Agreement with literature values proves this combination of chemistry and instrumentation provides a simple, yet robust solution for automated kinase profiling.

Introduction

Kinases are one of the most diverse enzyme families being studied today. Because of the large number of genetic mutations inherent within this family, and their role in cell signaling, kinases have been implicated in a large number of diseases, including cancer and diabetes. This has also caused kinase-targeted drug discovery efforts to continue to grow in importance among today's pharmaceutical companies.

As new lead compounds are identified for their primary target through screening efforts, it is becoming increasingly important for these compounds to be profiled against other kinases to determine any possible off-target effects that these compounds might have. This is primarily due to the non-specificity of ATP Competitive inhibitors. These compounds, as explained above, compete with ATP to bind at the ATP binding site of the kinase. Because of this, they are also more likely to attach to the ATP binding pocket of other non-target kinases. This can cause unplanned, adverse effects to the potential patient. Typically profiling efforts include testing multiple concentrations of lead compounds in an IC_{50} format against kinases within the same target class, as well as from other kinase families. This creates the need for a sensitive assay that can be used with a large number of kinases, as well as flexible, easy to use instrumentation that can be used for compound titration, low-volume reagent delivery, and signal detection. Promega's ADP-Glo™ Kinase Assay, when used together with BioTek's liquid handling and detection instrumentation create the ideal solution to meet the demands for automated kinase profiling.

ADP-Glo™ Kinase Assay

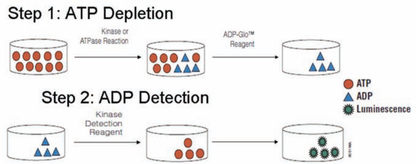

Figure 1 – Principle of the ADP-Glo™ Kinase Assay. The assay is performed in two steps: 1. After the kinase or ATPase reaction, ADP-Glo™ Reagent is added to terminate the kinase reaction and deplete the remaining ATP. 2. The Kinase Detection Reagent is added to convert ADP to ATP and allow the newly synthesized ATP to be measured using a luciferase/luciferin reaction. The light generated correlates to ADP present and kinase or ATPase activity.

BioTek Instrumentation

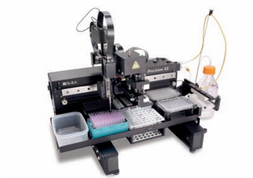

Figure 2 – Precision™ XS Microplate Sample Processor

The Precision™ XS combines a single-channel sample processing head, an 8-channel pipetting head and an 8-channel bulk reagent dispenser in one instrument. The instrument was used to serially titrate ATP, enzyme, and compounds across a 96-well microplate, as well as dispense assay components to the 384-well assay plates.

Figure 3 – Synergy™ MX Monochromator-Based Multi-Mode Microplate Reader

The Synergy™ Mx uses a dedicated optical system, separate from the fluorescence optics, for high-performance luminescence detection. An ultra low noise digital photon integration system and high-quality optics ensure the best sensitivity available today. The instrument was used to read the luminescent signal from all assay plates. The Luminescence Detection Method was chosen, and a 1.0 second integration time was used. Automatic Sensitivity Adjustment was used to detect the well on the plate containing the highest luminescent signal. All wells were appropriately adjusted to that well.

Automated Kinase Profiling Method

Precision™ XS

1. Transfer 80 µL of 3% DMSO from a 1 mL 96-well deepwell plate to columns 2-12 of a 96-well microplate.
2. Aspirate 20 µL from column 1, containing the highest concentration of test compound, dispense to column 2, and perform a 75 µL 10X mix.
3. Repeat the procedure for columns 2-11, leaving column 12 as a no compound control.
4. Transfer 5 µL in duplicate from each column of the titration plate to the 384-well assay plate.
5. Pre-mix 5X and transfer 5 µL of enzyme/substrate and ATP solutions to the appropriate compound wells.

   Shake the assay plate 30 seconds using an orbital shaker, and incubate for the appropriate time at RT
6. Pre-mix 5X and transfer 15 µL of ADP-Glo™ Reagent to all assay wells.

   Shake the assay plate 30 seconds using an orbital shaker, and incubate for 40' at RT
7. Pre-mix 1X and transfer 30 µL of Kinase Detection Reagent to all assay wells.

   Shake the assay plate 30 seconds using an orbital shaker, and incubate for the appropriate time at RT

Synergy™ MX

8. Read assay plate on Synergy™ MX reader.

Similar titration and reagent dispensing methods were also used during the assay optimization process. Volumes titrated ranged from 20-40 µL, while DMSO, enzyme/substrate, and ATP solution transfer volumes remained at 5 µL. ADP-Glo™ and Kinase Detection Reagent transfer volumes were the same as above.

Kinase Assay Optimization

In order to ensure proper pharmacology when performing kinase profiling experiments on lead compounds, it is imperative that each kinase assay has been previously optimized. These optimization experiments include ATP optimization, and enzyme optimization. Validation of assay performance can be achieved by determining Z'-Factor scores, as well as performing pharmacology studies using known kinase inhibitors. Five kinases were taken through this process, including Src, ZAP70, ROCK1, AKT2, and PI3 Kinase Alpha.

ATP Optimization

Assay sensitivity to weak inhibitors is improved by using [ATP] at $K_{m\ app}$. The same point is also true for ATP competitive inhibitors. This is an important consideration for profiling applications. Figure 1 demonstrates an example of the ATP titration for Src kinase. The $K_{m\ app}$ was determined to be 13.9 µM using the Michaelis-Menten equation. Table 1 lists the ATP $K_{m\ app}$ values for the other kinases tested.

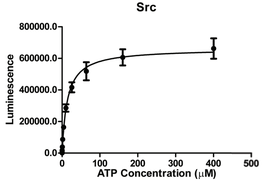

Figure 4 – ATP Titration for Src Kinase

Kinase Assay Optimization

Enzyme Optimization

Once $K_{m\ app}$ values are established, appropriate enzyme concentrations need to be determined. This is typically a balance of minimizing enzyme consumption (i.e. cost) and obtaining sufficient signal relative to background (SB) for adequate performance for profiling experiments. It is also important to be certain that the enzyme concentration chosen is within the initial rate velocity for the enzyme reaction. Enzyme titrations were performed in order to determine enzyme concentrations yielding SB levels between 5 and 20. Standard curves were also included to determine % ATP conversion. Confirmation of SB level at various individual enzyme concentrations was then performed, and the SB_{10} concentration determined. Figure 1 demonstrates the enzyme titration and confirmation for Src kinase. The SB_{10} enzyme concentration was determined to be 0.68 ng/rxn, which represents 4.71% ATP conversion. Table 1 lists the SB_{10} values for the other kinases tested.

Z'-Factor Validation

Z'-Factor validations were performed for each kinase using the SB10 concentration of enzyme. Selective inhibitors for the 5 kinases were identified (Table 1). Twenty-four replicates of 0 and 10 µM inhibitor were used for the positive and negative controls. Z' factors generated, which ranged from 0.75 to 0.89 (Table 1), confirm that each of the optimized kinase reactions are robust, and should also yield accurate pharmacological results when used in a kinase profiling setting.

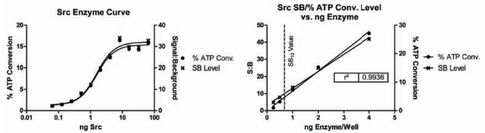

Figure 5 – Enzyme titration and confirmation graphs for Src Kinase.

Assay Optimization and Validation Data

Kinases	ATP Titration: ATP $K_{m\ app}$ (µM)	Enzyme Titration: Enzyme (ng/rxn)	Enzyme Titration: SB Level	Enzyme Confirmation: SB_{10} Enzyme (ng/rxn)	Enzyme Confirmation: ATP Conversion (%)	Z'-Factor Validation: Selective Inhibitor	Z'-Factor Validation: Z'-Factor Value
Src	13.9/50[1]	0.25	4.5	0.68	4.71	PP2	0.77
		1.015	12.3				
		2.03	19.8				
ZAP70	1.316/2[1]	6.25	5.7	11.3	18.5	Staurosporine	0.83
		12.5	11.2				
		25	20.5				
ROCK1	4.616/3[1]	6.25	3.8	11.5	8.26	Y27632	0.75
		12.5	8.3				
		25	15.6				
AKT2	225.9/200[1]	2.03	5.5	4.12	3.37	AKT1/2 Kinase Inhibitor	0.82
		4.06	9.8				
		16.25	23				
PI3 Kinase Alpha	26.41/25[1]	1.02	5.6	3.43	7.47	Wortmannin	0.89
		2.03	11.2				
		4.06	23.2				

[1]ATP Km app values for Invitrogen SelectScreen® Kinase Profiling Service

Table 1 – Assay optimization and Z'-Factor validation results for the optimized kinase reactions.

Universal Kinase Inhibitor Validation

Pharmacology validation of the assay is an important consideration, especially for profiling applications. Staurosporine is a potent, but non-selective kinase inhibitor due to the fact that it directly binds to ATP-binding sites which are largely conserved through the kinome. Thus it is a useful tool to perform an intial assessment of pharmacology for kinase assays. Results were as expected, as IC_{50} values for Src, ZAP70, ROCK1, and AKT2 were all below 15 nM. PI3 Kinase Alpha, being a lipid kinase, reacts differently with staurosporine and will possess IC_{50} values generally orders of magnitude higher than for kinases with non-lipid substrates. The IC_{50} value, 2 µM, also closely parallels what has been seen with staurosporine and the PI3 kinase family.

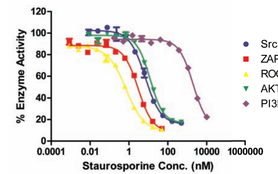

Figure 6 – Inhibition curves for Staurosporine with Src, ZAP70, ROCK1, AKT2, and PI3 Kinase Alpha

Kinase Profiling

The five optimized and validated kinase assays, along with 4 others previously optimized (IKK Alpha, PKA, PKC, and DNA PK), were profiled using Wortmannin as the model lead compound of interest. This compound, being a specific inhibitor for PI3 Kinase, generates IC_{50} values typically in the low nM range for this kinase. At higher concentrations, this compound has also shown inhibitory effects on DNA PK. Results once again agreed with expected results, with IC_{50}s for PI3 Kinase Alpha and DNA PK being 0.15 and 7.1 nM, respectively. Wortmannin showed little or no effect on the remaining protein Tyrosine or Serine/Threonine Kinases.

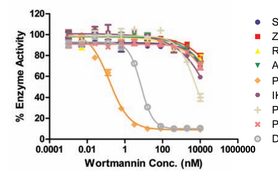

Figure 7 – Wortmannin Profiling Data.

Conclusions

1. The Precision™ XS provides an easy to use solution to deliver accurate compound titrations for IC_{50} determination.
2. The Precision™ XS can consistently deliver all assay components in volumes as low as 5 µL.
3. The ultra low noise integration system, and high-quality optics in the Synergy™ MX allow for dependable luminescent reads in 384-well format.
4. Promega's ADP-Glo™ Kinase Assay is flexible and sensitive enough to be used with a wide range of kinases that have varying ATP $K_{m\ app}$ values.
5. The combination of BioTek's instrumentation, and Promega's ADP-Glo™ Kinase Assay creates an ideal solution for high-density, automated kinase profiling.