# Rapid, High-Throughput Screening of Protein Kinase Inhibitors Using the ProteOn™ XPR36 Protein Interaction Array System

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## Introduction

Drug discovery and development expend considerable effort to identify and characterize molecules that have very high affinity and specificity for their intended protein targets, as well as low cross-reactivity and toxicity. The drug discovery workflow includes a screening component wherein highly sensitive, reproducible binding measurements are an absolute requirement. Binding assays must be developed quickly to respond to the changing needs of the development process while allowing high throughput to be maintained.

The importance of the mitogen-activated protein kinases (MAPKs) ERK2 and p38 in cell signaling makes these enzymes particularly interesting as drug targets during the screening phase of drug discovery. ERK2 has been implicated in the control of cell proliferation, differentiation, and survival (Saba-El-Leil et al. 2003). It has also been shown to have an essential function in embryonic development (Krens et al. 2008). Stress-activated p38 has been shown to play a role in breast cancer metastasis; it is highly expressed in samples from patients with lymph node metastasis vs. samples from patients without metastatic disease (Bai-lin et al. 2006). In addition, p38 regulates expression of many cytokines and thus has an important role in the activation of the immune response. MAPKs such as ERK2 and p38 are increasing in popularity as molecular targets for drug development, and their inhibitors are part of many novel drug screens used to identify new drugs (English and Cobb 2002).

Surface plasmon resonance (SPR) provides real-time data on affinity, specificity, and kinetics without the need for costly labels and as such is a tool widely used to study biomolecular interactions. However, to be useful to the drug discovery screening process, interaction studies using SPR must be fast and cost effective to develop and be amenable to high throughput. Traditional SPR systems that run kinetic analyses sequentially cannot provide the required throughput or speed of assay development.

The ProteOn XPR36 protein interaction array system and One-shot Kinetics™ approach expand the capabilities of the traditional SPR workflow by enabling multiple quantitative binding experiments to be run in parallel. The ProteOn system integrates a unique 6 X 6 interaction array for the analysis of up to six ligands with up to six analytes to produce 36 data points simultaneously. Multiplexing enables rapid screening of several putative inhibitors with several kinases at a single inhibitor concentration to identify possible “hits.” It then provides the ability to generate complete kinetic profiles for the interaction of several hits with specific kinases simultaneously.

In this report, we demonstrate the rapid optimization of immobilization conditions for the protein kinase targets which ensure that the bound protein remains in an active state. Compounds from two small-molecule kinase inhibitor libraries were also screened against each target kinase to identify hits, and a detailed kinetic binding analysis of the positive hit compounds was conducted to determine their binding constants. Screening of 110 compounds took only 5 hours, and all the work was completed using a single sensor chip, without the need for multiple methods or instruments.

## Methods

### Instrument and Reagents

Experiments were performed using the ProteOn XPR36 system with ProteOn GLH sensor chips. The running buffer for kinase immobilization was ProteOn PBS/Tween (phosphate buffered saline, pH 7.4, 0.005% Tween 20). The buffer used to prepare the analytes, as well as the running buffer for the interaction of inhibitors with the kinases, was 50 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, 1 M $MnCl_2$, 5% DMSO, 0.005% Tween. All experiments were performed at 25°C. The ProteOn amine coupling reagents used were EDAC (N-ethyl-N’-(3-dimethylaminopropyl) carbodiimide), sulfo-NHS (N-hydroxysuccinimide), and 1 M ethanolamine hydrochloride solution, pH 8.5. The immobilization buffer was 10 mM sodium acetate, pH 5.5. Proteins p38 and ERK2 were gifts from Professor David Engelberg, Hebrew University of Jerusalem. Two kinase inhibitor libraries were obtained from EMD Chemicals, Inc.

### Immobilization of Protein Kinases

Four vertical channels were activated on the GLH chip using 200 µl of 0.2 M EDAC, 0.05 M S-NHS at a flow rate of 25 µl/min. The p38 and ERK2 kinases were diluted into acetate buffer pH 5.5, and immobilized onto the chip using standard amine coupling at a flow rate of 25 µl/min for 5 min. The p38 (50 µg/ml) was immobilized in the first

two channels, with and without the addition of the small-molecule inhibitor SB203580 (10 µM). The ERK2 kinase (50 µg/ml) was immobilized in the other two channels, with and without the addition of ATP (1 mM) and $MgCl_2$ (2 mM). All four channels were deactivated with an injection of 150 µl of 1 M ethanolamine-HCl (25 µl/min).

#### Confirmation of Protein Kinase Activity

Three analytes were injected (p38 inhibitors SB203580 and PD169316 and the ERK2 substrate ATP) using a concentration series of 50 mM to 1.56 mM, in twofold dilution steps. They were injected in the horizontal direction at a flow rate of 25 µl/min. Association was monitored for 1 min and dissociation was monitored for 2 min. Kinetic and equilibrium measurements were calculated for each interaction.

#### Screening

The two protein kinase inhibitor libraries were diluted to 5% DMSO and injected at a single concentration (50 µM) in the horizontal direction at a flow rate of 25 µl/min. This configuration enabled the testing of six different inhibitors in a single analyte injection. Association was monitored for 1 min and dissociation for 2 min. A total of 110 inhibitors were screened. The binding response of each inhibitor was determined at the same time point during the association phase and exported into a report point table. Response was normalized by dividing the response level by the molecular weight of the inhibitor. Those with the highest normalized response, above the 0.4 threshold (arbitrarily chosen for this study), were subjected to full kinetic and equilibrium analyses using the same concentration series as described earlier.

#### Data Analysis

The data were analyzed with ProteOn Manager™ version 2.1 software. Binding curves were processed for baseline and start injection alignment, together with channel reference subtraction using a protein with no affinity for kinase inhibitors (rabbit IgG). EVC correction, a data manipulation process for correcting an artifact caused when DMSO is used (Bio-Rad Protocol Guide 5822B) was also applied. Each set of six reference-subtracted sensorgrams was fitted globally to curves describing a homogeneous 1:1 bimolecular reaction model. Each kinase ligand surface interacting with its different analyte concentrations was grouped together to fit the $k_a$, $k_d$, and $R_{max}$ parameters, and the equilibrium dissociation constant, $K_D$, was calculated using the equation $K_D = k_d / k_a$. In most cases where binding events were too fast, however, only equilibrium analysis was performed to determine the affinity constant $K_D$ directly. In the screening mode, the software performed a report point analysis and compiled a table in which the response was listed for each putative inhibitor at a fixed analyte concentration. After normalization for molecular weight, this data was used to select the inhibitors with the highest binding response.

## Results and Discussion

#### Immobilization of Protein Kinases

The two kinases, p38 and ERK2, were immobilized on a GLH chip. Immobilization conditions to the chip surface were optimized to maintain maximal kinase activity, as the analytes to be tested are small molecules. The kinases were immobilized with and without preincubation with a standard inhibitor in order to protect their binding sites (Figure 1). The level of ligand immobilization was significantly higher for p38 with preincubation, and immobilizing this kinase without preincubation completely abolished its ability to bind either of two standard inhibitors (Figure 2). Preincubation with inhibitor SB203580 protected the binding site and therefore maintained its activity to bind and interact with both inhibitors, for which the binding constants were determined.

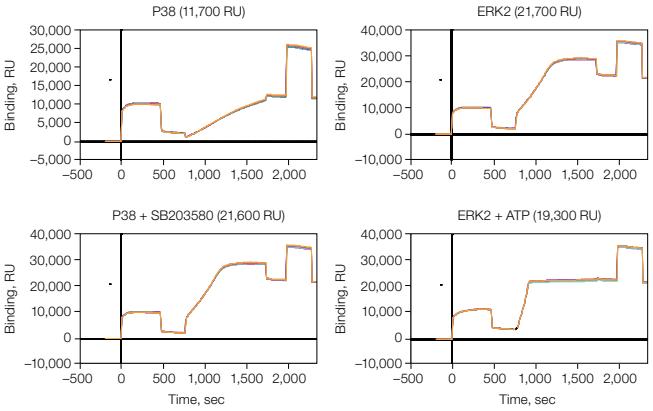
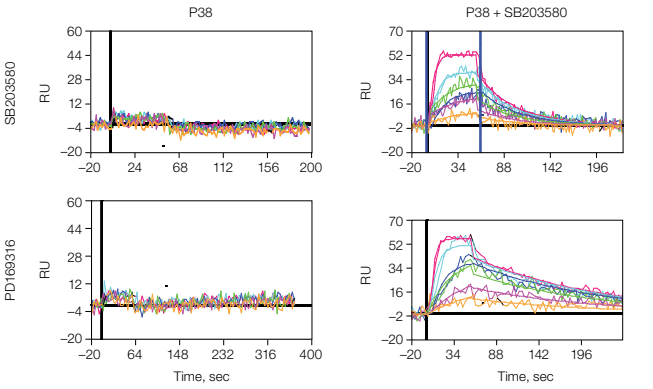


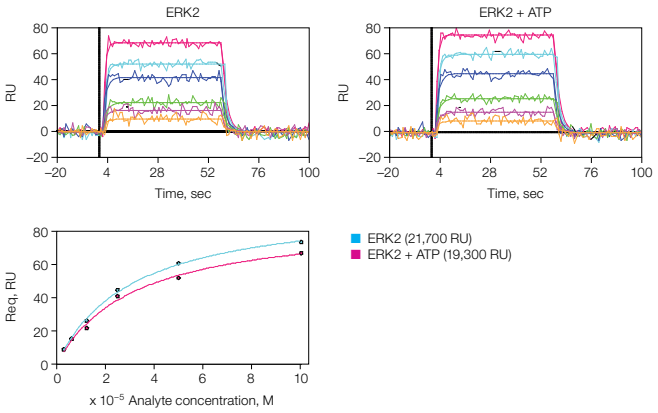

**Fig. 1. Immobilization of p38 and ERK2.** The two kinases were immobilized on a GLH chip, without (upper panels) and with (lower panels) preincubation with an appropriate analyte (inhibitor SB20358 for p38 and ATP for ERK2). The response units (RU) for the amount of each kinase bound to the chip are given for each case. Immobilization conditions were optimized for maximal binding and activity of the kinases. The number in the upper left corner of each sensorgram denotes the channel in which the data was recorded.




| Analytes | Ligand | $k_a$, $M^{-1}s^{-1}$ | $k_d$, $s^{-1}$ | $K_D$, M | $R_{max}$, RU |
|---|---|---|---|---|---|
| **SB203580** | P38 (11,700 RU) | No binding | | | |
| | P38 + SB203580 (21,600 RU) | $6.93 \times 10^5$ | $2.22 \times 10^{-2}$ | $3.21 \times 10^{-8}$ | 43 |
| **PD169316** | P38 (11,700 RU) | No binding | | | |
| | P38 + SB203580 (21,600 RU) | $4.64 \times 10^5$ | $5.79 \times 10^{-3}$ | $1.25 \times 10^{-8}$ | 42 |

**Fig. 2. Immobilizing in the presence of analyte to preserve p38 activity.** Two inhibitors of p38 (SB203580 and PD169316) were subjected to a binding analysis with p38 immobilized to a GLH chip in the absence (left hand panels) or presence (right hand panels) of inhibitor. Immobilization of p38 without preincubation with the inhibitor totally abolished its ability to bind either inhibitor, while preincubation provided active immobilized p38 which could be used for a complete binding analysis with both inhibitors. The response units (RU) for the amount of p38 bound to the chip are given for each case.

The kinase ERK2 was also immobilized with and without preincubation with ATP, a known substrate for this kinase. There was no significant difference in immobilization levels, with and without preincubation. Both kinetic and equilibrium analyses were then performed for the interaction between ERK2 and ATP, and the kinetic and affinity constants were similar, whether or not preincubation with ATP was performed (Figure 3). Therefore the binding site is not affected by immobilization, which was then performed without preincubation with ATP for all subsequent experiments.




| Analytes | Ligand | Equilibrium Analysis $K_D$, M | Equilibrium Analysis $R_{max}$, RU |
|---|---|---|---|
| ATP | ERK2 (21,700 RU) | 3.16 x $10^{-5}$ | 88 |
| | ERK2 + ATP (19,300 RU) | 2.99 x $10^{-5}$ | 95 |

**Fig. 3. Immobilizing ERK2 in the presence or absence of ATP.** ERK2 was immobilized on a GLH sensor chip in the presence or absence of ATP. Preincubation with ATP had no impact on the activity of the immobilized ERK2 to bind ATP, as the kinetic and equilibrium analyses of the interaction of the immobilized ERK2 with ATP yielded virtually identical values for both cases. The response units (RU) for the amount of ERK2 bound to the chip are given for each case. The top panel shows the five sensorgrams for the six concentrations of each compound. The middle panel shows the plot of equilibrium response versus analyte concentration, and the bottom table lists the equilibrium constants derived from the plots.

### Screening of Two Inhibitor Libraries

The screening assay was performed on two standard kinase inhibitor libraries using a single concentration of each small-molecule inhibitor. This configuration enabled the testing of six different inhibitors in a single injection. The signals were collected and processed followed by a report point analysis in which the binding signal level for each compound was determined at the same time point and overlaid. In order to properly compare binding affinities of the inhibitors, the signal obtained from every inhibitor was normalized by dividing the signal level with the corresponding molecular weight. An arbitrary threshold was set for which all normalized signals above it were considered hits (Figure 4). These hits were then subjected to detailed kinetic and equilibrium analyses using six analyte concentrations (representatives can be seen in Figures 5 and 6). The kinetic and affinity constants of the inhibitors showing the strongest binding could then be compared to select the inhibitor with the proper characteristics for the target drug application.

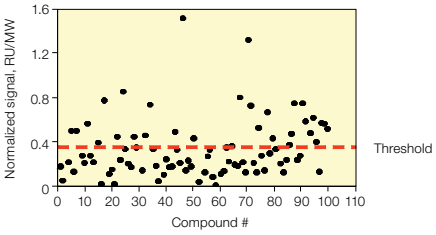
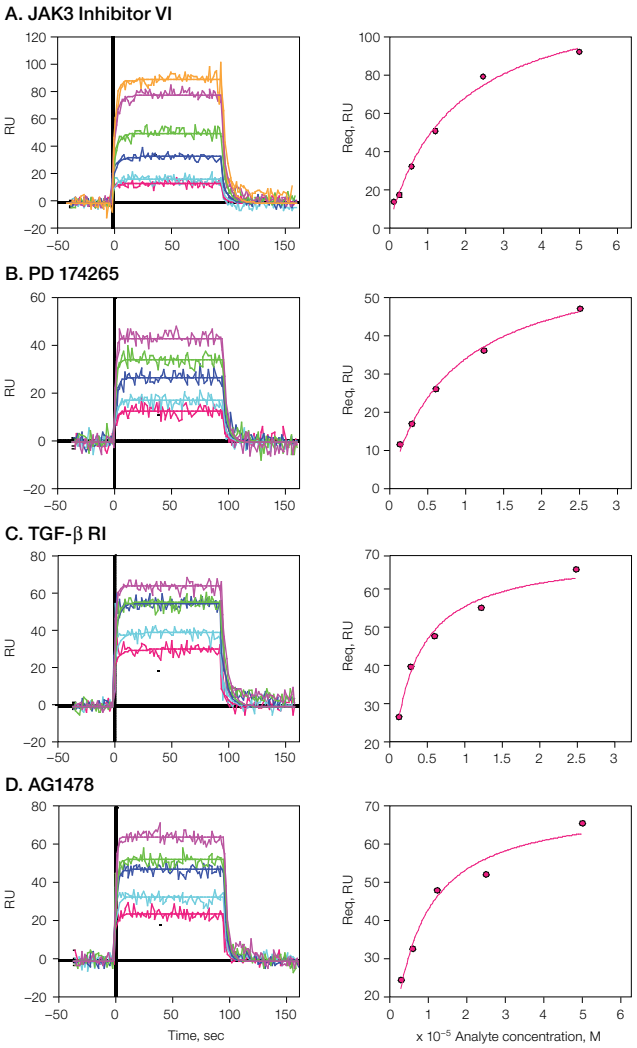


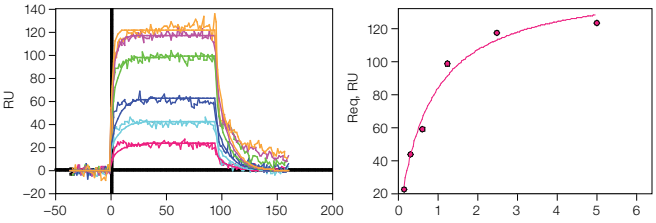

**Fig. 4. Normalized report point analysis of inhibitor binding.** The binding responses obtained in the report point analysis were divided by molecular weight and plotted versus the compound number. An arbitrary threshold value was set to define positive hits for putative inhibitory compounds.



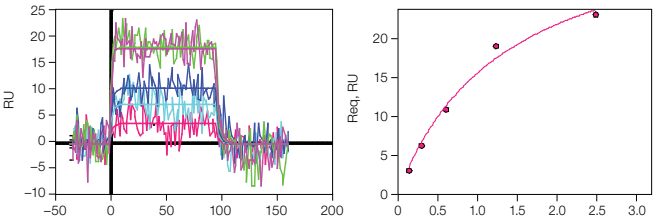

| | Equilibrium Analysis $K_D$, M | Equilibrium Analysis $R_{max}$, RU |
|---|---|---|
| A. JAK3 Inhibitor VI | 1.73 x $10^{-5}$ | 126 |
| B. PD 174265 | 8.16 x $10^{-6}$ | 61 |
| C. TGF-β RI | 2.57 x $10^{-6}$ | 69 |
| D. AG1478 | 3.25 x $10^{-6}$ | 71 |

**Fig. 5. Detailed binding analysis of several inhibitor hits for p38 kinase.** Four putative inhibitors with high binding response as identified in a normalized point analysis were then subjected to equilibrium analysis using six analyte concentrations. The left panels show the five sensorgrams for the six concentrations of each compound. The right panels show the plot of equilibrium response versus analyte concentration, and the bottom table lists the equilibrium constants derived from the plots.

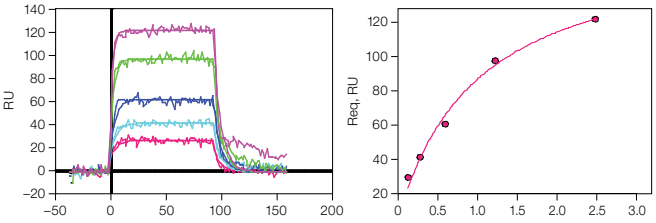
A. JAK3 Inhibitor VI



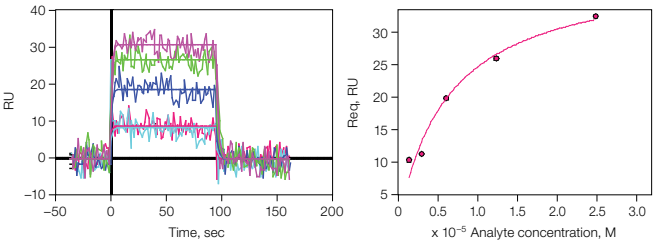

B. PD 174265




C. Staurosporin




D. AG1478




| | Equilibrium Analysis | |
|---|---|---|
| | $K_D$, M | $R_{max}$, RU |
| **A. JAK3 Inhibitor VI** | $7.68 \times 10^{-6}$ | 148 |
| **B. PD 174265** | $1.34 \times 10^{-5}$ | 36 |
| **C. Staurosporine** | $9.79 \times 10^{-6}$ | 170 |
| **D. AG1478** | $6.21 \times 10^{-6}$ | 40 |

**Fig. 6. Detailed binding analysis of several inhibitor hits for Erk2 kinase.** Four putative inhibitors with high binding response as identified in a normalized point analysis were then subjected to equilibrium analysis using six analyte concentrations. The left panels show the five sensorgrams for the six concentrations of each compound. The right panels show the plot of equilibrium response versus analyte concentration, and the bottom table lists the equilibrium constants derived from the plots.

## Conclusions

One hundred and ten small-molecule inhibitors in two standard kinase inhibitor libraries were screened rapidly (5 hours) against two important drug targets (p38 and ERK2), using the ProteOn XPR36 system and One-shot Kinetics technique. Optimization of the immobilization conditions, confirmation of activity, screening of the small-molecule inhibitor libraries, and full kinetic analysis of hits were all performed using a single GLH sensor chip. The ProteOn XPR36 system is thus a valuable tool for drug discovery and development, enabling the rapid screening of small-molecule libraries and generation of full kinetic data for thorough analysis of the binding characteristics of hits, without the need for multiple platforms and analytical methods. Multiplexed SPR is an indispensable asset across the drug discovery workflow, including evaluation of candidate lead compounds (Bronner et al. 2008a), rapid assay development (Bronner et al. 2008b), rapid screening and selection of antibody capturing agents (Bronner et al. 2008c), and high-throughput kinetic analysis and affinity determination of monoclonal antibodies (Nashol et al. 2008).

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