Simplifying Kinase Profiling Using ADP Detection with the Transcreener™ Kinase Assay

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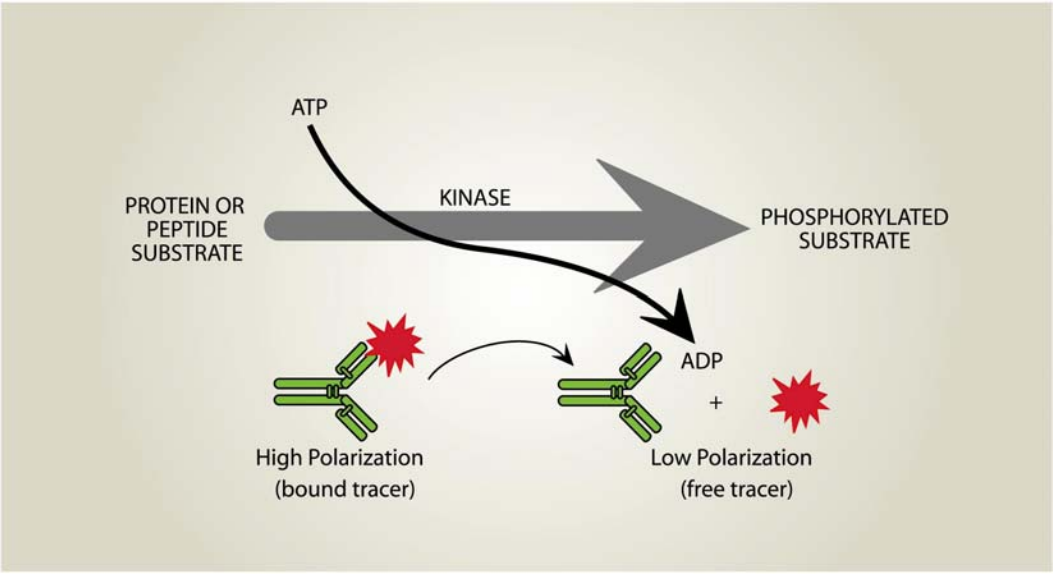
Introduction

A variety of HTS kinase assays rely on detection methods specific for the single phosphorylated product. This is problematic in that each of the many kinase subfamilies must then have it's own unique set of assay reagents, specifically optimized. To eliminate these difficulties, BellBrook Labs has developed the universal Transcreener™ Kinase Assay, a homogeneous fluorescent polarization HTS assay that directly detects ADP, the common product in all kinase reactions. A single set of reagents, which includes a novel anti-ADP monoclonal antibody and a red-shifted ADP tracer, can be used to assay across the entire protein kinase family. Rapid generation of meaningful and comparable data is obtained due to standardized assay methodologies and data analyses. The Transcreener™ Kinase Assay is not dependent on a modified acceptor substrate, and can therefore be used with proteins or peptides at various concentrations. Likewise, the assay can be easily optimized to accommodate a selection of ATP concentrations. Unlike other generic assays, excellent Z' values are obtained at 10-30% ATP conversions. In this study the Transcreener™ Kinase Assay was used to profile 12 known kinase inhibitors with PKA, cdk5/p35, and Abl kinases. Our results indicated that the Transcreener™ Kinase Assay correctly identified specific inhibitors unique to each kinase and demonstrated correct pharmacology. This single powerful assay platform can be used to profile kinase proteins, substrates, and inhibitors leading to reduced development costs and accelerated drug discovery.

Figure 1.

Transcreener™ Kinase Assay Principle

ADP produced during transfer of phosphate to acceptor substrate is detected using a competitive fluorescence polarization immunoassay. Because it relies on detection of the invariant reaction product, the same detection reagents can be used for any kinase and any protein or peptide substrate.



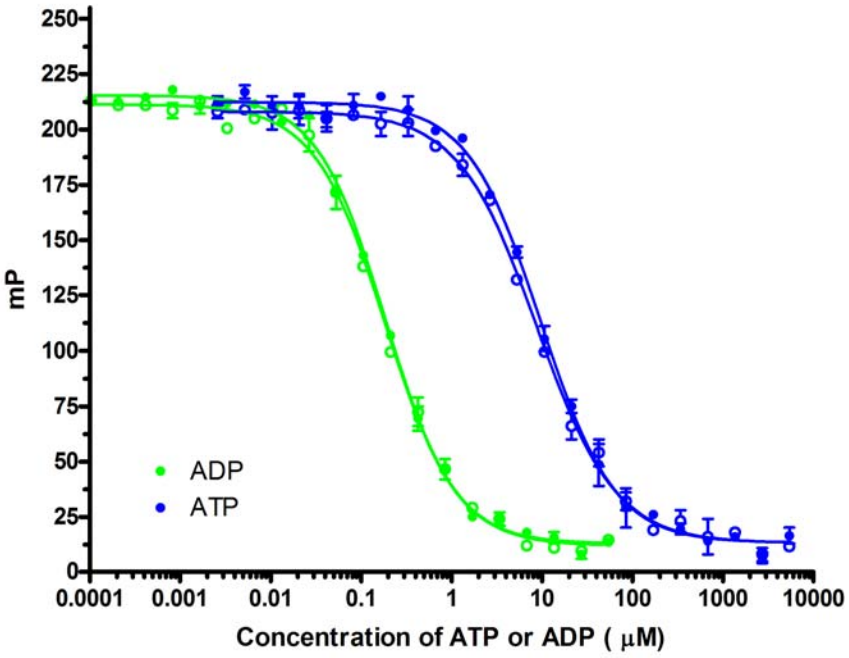
Materials and Methods

In general, the Transcreener™ Kinase Assay consisted of a one hour, 25°C kinase reaction (20 µL), which was initiated with the addition of ATP/MgCl2. The kinase reaction was then stopped and the ADP detected by adding 20 µL of the Transcreener™ ADP-detection mix, bringing the total volume to 40 µL. Kinase reaction conditions: 50 mM HEPES (pH 7.5), 4 mM MgCl2, 2 mM EGTA, 1-200 µM ATP, 50 µM peptide substrate or 0.1 mg/mL Histone H1 protein substrate. Transcreener™ ADP Detection Mix: 50 mM HEPES (pH 7.5), 400 mM NaCl, 20 mM EDTA, 0.02% Brij-35, 5.3-111 µg/mL Transcreener™ Anti-ADP mAb, and 4 nM Transcreener™ AlexaFluor®-633 Tracer. All kinases and kinase substrates were purchased from Upstate, except PKA (Invitrogen). Kinase inhibitors, and basic buffer components were purchased from Sigma or Fisher. Solvent tolerance studies showed that the Transcreener™ Detection Mix is tolerant to DMSO, DMF, ethanol, and acetonitrile at 20%, 6%, 16%, and 10%, respectively. Tecan Ultra settings and filters: Ex612nm (10 nm bandwidth), Em670nm (25 nm bandwidth).

The free tracer reference was set to 20 mP, and complete buffer devoid of tracer (containing mAb) was used as a blank for both the sample and reference wells. Assays were performed in black Corning (catalog #3654) flat-bottom 384-well plates.

Figure 2.

Transcreener™ Monoclonal Antibody: High Selectivity for ADP



The Transcreener™ monoclonal antibody is a novel anti-ADP mAb that can distinguish between a single phosphate group. Antibody/tracer complex was mixed with increasing concentrations of ATP or ADP to generate competition curves. The anti-ADP mAb is approximately 50x more selective for ADP than ATP yielding EC_{50} values of 0.2 µM and 9.7 µM, respectively. AMP does not have a significant affinity for the anti-ADP mAb (data not shown). The polarization signal is stable between 1 hour (closed circles) and 5 hours (open circles).

Figure 3.
Transcreener™ Kinase Assay: Flexibility for a wide range of ATP Concentrations

The Transcreener™ Kinase Detection Module can be used over a wide range of ATP concentrations. In an attempt to mimic ADP generated during a kinase reaction, standard curves optimized for 1 µM, 10 µM and 200 µM ATP were prepared by keeping the adenosine concentration constant. The IC50 values for the 1 µM, 10 µM, and 200 µM ATP-ADP curves are 0.3 µM, 1.3 µM, and 20 µM ADP, respectively, showing that a low concentration of ADP is capable of producing significant shifts in mP values.

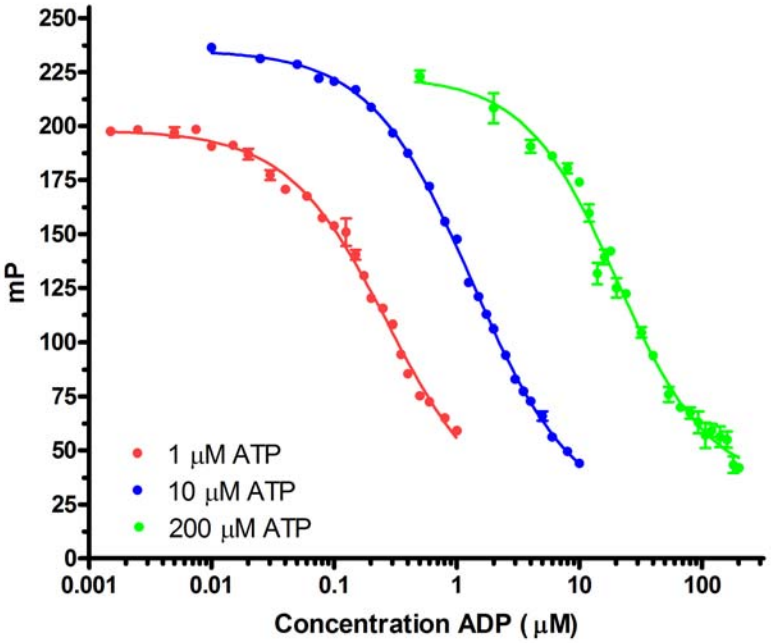


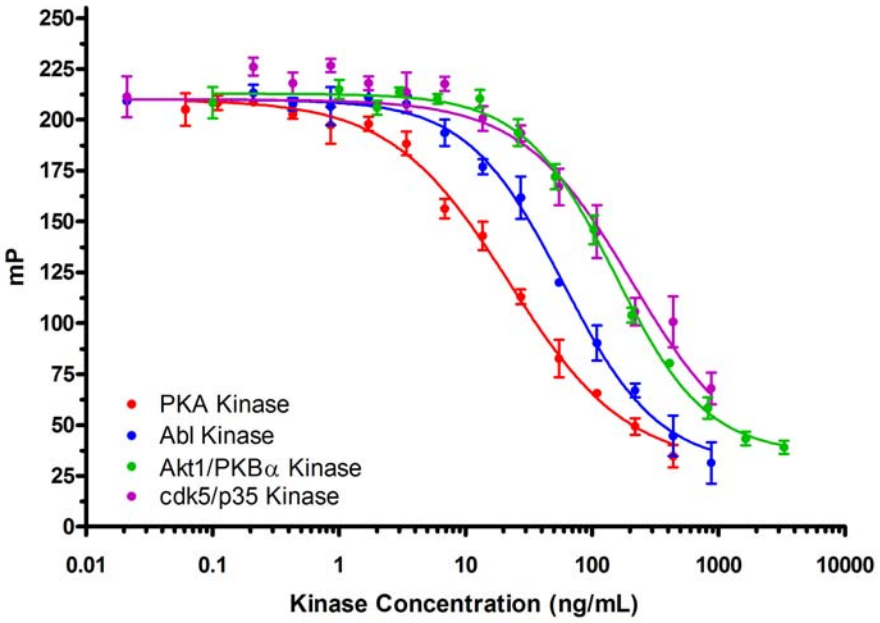
Table 1.
Excellent Z' Values at Low ATP % Conversion

% Conversion	mP Shift	Z'
0	0	0.00
0.25	5	-6.62
0.5	8	-3.82
0.75	14	-1.16
1	16	-1.43
1.5	19	-0.89
2	28	-0.06
3	40	0.06
4	49	0.31
6	64	0.46
8	81	0.58
10	89	0.61
12.5	109	0.67
15	115	0.75
17.5	124	0.74
20	130	0.77
25	142	0.76
30	153	0.76
35	159	0.80
40	164	0.81
50	170	0.75
60	180	0.85
80	187	0.81
100	192	0.85

The Transcreener™ Kinase Detection Module is robust at less than 10% ATP conversion. The Z' factor was calculated at various % ATP conversions using the 10 µM ATP standard curve data points (n=15). Excellent Z' values of 0.45 to 0.85 were observed over the 6-100 % ATP conversion range.

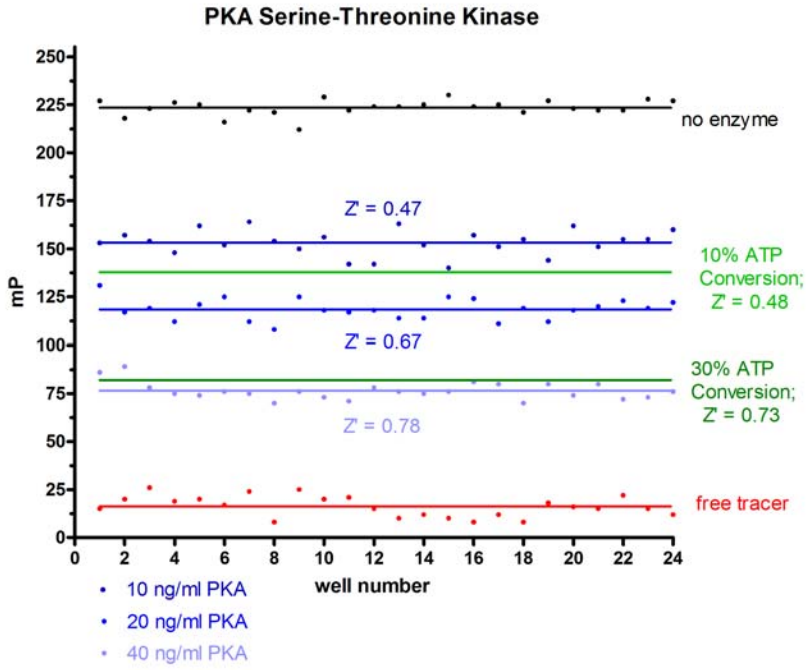
Figure 4.
Transcreener™ Kinase Assay: One Assay, Any Kinase, Any Substrate

A single set of reagents can be used to assay across kinase families using peptide or protein substrates. Enzyme titration curves for three Ser/Thr kinases and one Tyr kinase were prepared by adding an equal volume of the Transcreener™ ADP-detection mix after the one hour 25°C kinase reaction. Kinases were serially diluted two-fold in buffer containing the following substrates: PKA = kemptide; Abl=Abltide, cdk5/p35 =Histone H1, and Akt1/PKBalpha =Akt/SGK substrate peptide (see Materials and Methods).



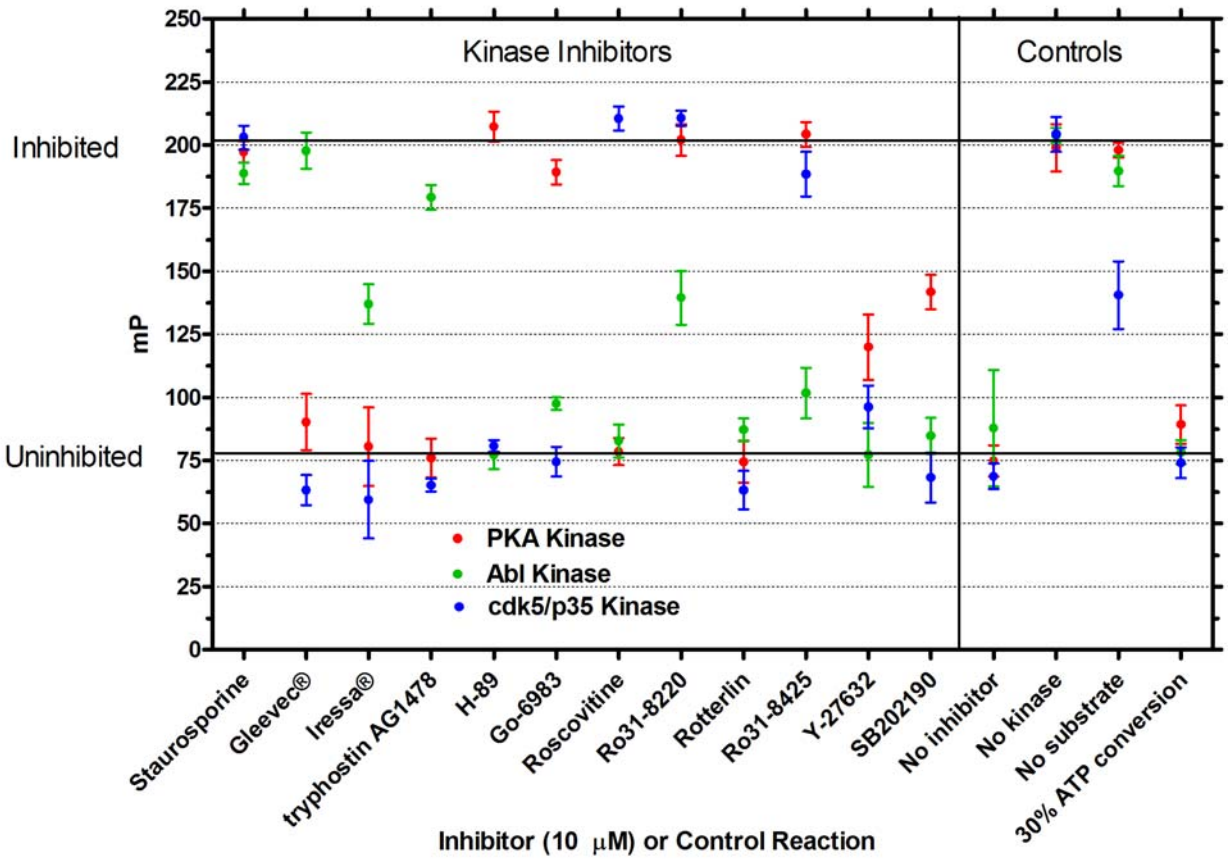
The kinase titration curves were used to estimate the enzyme requirement for subsequent experiments.

Figure 5.
Transcreener™ Kinase Assay: Robust Detection in Linear Range



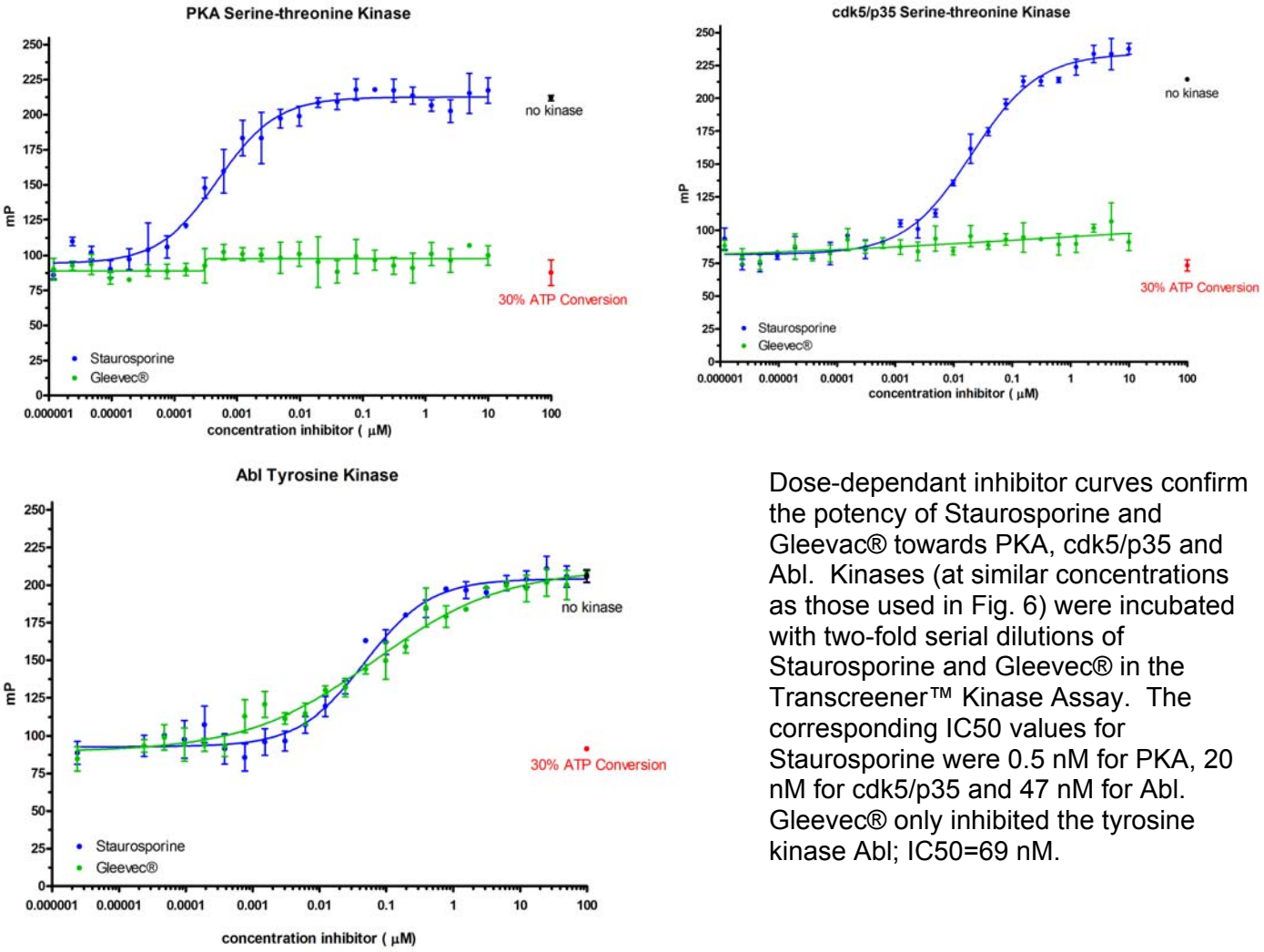
A robust ADP-detection module leads to a robust Transcreener™ Kinase Assay. Kinase reactions containing 10 µM ATP and 10-40 ng/mL PKA were incubated for one hour at 25°C as described in the Materials and Methods. The resulting Z' values demonstrate a high quality kinase assay at low % ATP conversion. Lines represent the means of the 24 data points. The green lines correspond to 10% and 30% ATP conversion.

Figure 6.
Transcreener™ Kinase Assay: Accelerate Inhibitor Pharmacology



Profiling known inhibitors with PKA, cdk5/p35, and Abl kinases demonstrates correct pharmacology using the Transcreener™ Kinase Assay. A panel of twelve kinase inhibitors at 10 µM (1% DMSO) were incubated with 10 µM ATP and 40 ng/mL PKA, 80 ng/mL Abl, or 240 ng/mL cdk5/p35 in the Transcreener™ Kinase Assay. At these enzyme concentrations, approximately 30% of the ATP was converted to ADP. As expected, Staurosporine inhibited all three kinases, while Rotterlin did not inhibit kinase activity. Gleevec® and Tryphostin AG1478 specifically inhibited Abl, whereas H89 and Go-6983 were selective towards PKA. Roscovitine only inhibited cdk5/p35. As evidenced by a decrease in the mP value, ATP was non-productively hydrolyzed by cdk5/p35 in the absence of Histone H1. The inhibitor panel also identified correct pharmacology for Akt/PKBalpha kinase at 75 µM ATP, the ATP Km for that enzyme (data not shown).

Figure 7.
Dose Dependent Inhibitor Curves Confirm the Potency of Staurosporine and Gleevec®



Dose-dependant inhibitor curves confirm the potency of Staurosporine and Gleevac® towards PKA, cdk5/p35 and Abl. Kinases (at similar concentrations as those used in Fig. 6) were incubated with two-fold serial dilutions of Staurosporine and Gleevec® in the Transcreener™ Kinase Assay. The corresponding IC50 values for Staurosporine were 0.5 nM for PKA, 20 nM for cdk5/p35 and 47 nM for Abl. Gleevec® only inhibited the tyrosine kinase Abl; IC50=69 nM.

Conclusions:

1. A novel monoclonal antibody discriminates between one phosphate group and binds ADP selectively.

2. Transcreener™ Kinase Assay can be optimized to accommodate various ATP concentrations (1 µM to >100 µM).

3. A single set of reagents can be used to assay multiple kinases with diverse acceptor substrates.

4. The Transcreener™ Kinase Assay is sensitive and robust yielding Z'>0.5 at > 6% ATP conversion.

5. Pharmacological relevance of 12 known kinase inhibitors with two Ser/Thr and one Tyr kinase has been established with the Transcreener™ Kinase Assay.

This work was supported by NIH SBIR grant CA110535-01A1. Transcreener™ Assay Platform is patent pending. Transcreener™ is a trademark of BellBrook Labs. AlexaFluor® is a registered trademark of Molecular Probes, Inc (Invitrogen). Gleevec® is a registered trademark of Novartis. Iressa® is a registered trademark of AstraZeneca. Brij® is a registered trademark of ICI Americas, Inc.