A Robust and Flexible HTS Kinase Assay Using ADP Detection

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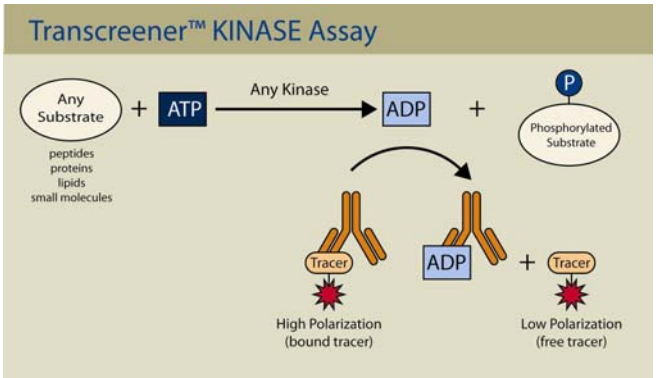
Introduction

Conventional 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, competitive fluorescent polarization HTS assay that directly detects ADP, the invariant reaction product of all kinase reactions. A single set of reagents, which includes a novel anti-ADP antibody and a far red ADP-Alexa Fluor® 633 tracer, can be used to assay across the entire protein kinase family as well as the lipid kinase and metabolic kinase families. 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 therefore is not limited to use with traditional kinase enzymes, but can be used to assay all ADP-producing enzymes, such as ATPases. Likewise, the assay can be easily optimized to accommodate a range of ATP concentrations. Excellent Z' are obtained at >8% ATP conversions. In one study the Transcreener™ KINASE Assay was used to screen the Gen-Plus compound library against PKA. Furthermore, the assay was used to specifically profile 12 known inhibitors with PKA, cdk5/p35, and Abl kinases. Our results indicate that the Transcreener™ KINASE Assay is robust and can correctly identify specific inhibitors unique to each kinase and demonstrates correct pharmacology. Use of a far red tracer significantly reduces compound interference. This single powerful assay platform can be used to profile kinase proteins, substrates, and inhibitors leading to reduced development costs, increased assay flexibility and accelerated drug discovery.

Figure 1. Transcreener™ Kinase Assay Principle

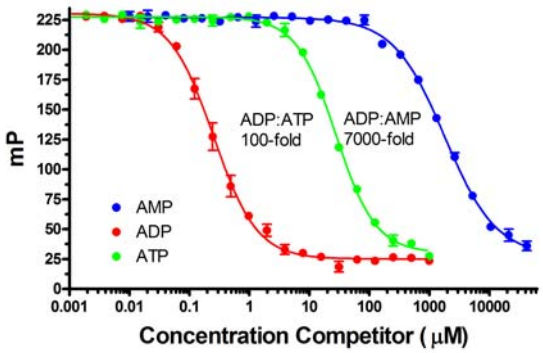


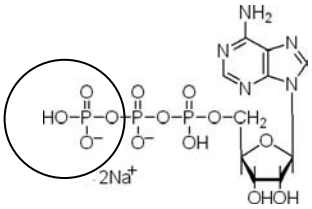
A far red, competitive fluorescence polarization immunoassay detects ADP produced during the transfer of phosphate (from ATP) to acceptor substrate. Because the Transcreener™ KINASE Assay technology relies on detection of the invariant reaction product, a single set of reagents can be used for any kinase and any substrate. The Transcreener™ KINASE Assay is not limited to use with traditional kinase enzymes, but can be used to assay all ADP-producing enzymes, such as ATPases.

Materials and Methods

Materials and Methods: Kinases and kinase substrates were purchased from Upstate, Invitrogen, Carna Biosciences, Sigma, or BPS Bioscience. Kinase inhibitors, and basic buffer components were purchased from Sigma or Fisher. The Alexa Fluor® 633 succinimidyl ester was purchased from Molecular Probes and the Gen-Plus Compound Library from Microsource Discovery Systems. In general, the Transcreener™ KINASE Assay consisted of a one hour, 25°C kinase reaction (10 µL), which was initiated with the addition of ATP/MgCl2. The kinase reaction was then stopped and the ADP detected by adding 10 µL of the Transcreener™ ADP detection mix, bringing the total volume to 20 µL. Kinase reaction conditions: 50 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 2 mM EGTA, 1-500 µM ATP, 50 µM peptide substrate, 3-10 µM protein substrates (ATF2, Histone H1, MEK1, or MBP), 20-50 µM lipid substrate, and 100 µM glucose or fructose-6-phosphate substrates. Transcreener™ ADP detection mix: 50 mM HEPES (pH 7.5), 400 mM NaCl, 20 mM EDTA, 0.01% Brij-35, 5-260 µg/mL Transcreener™ Anti-ADP mAb, and 4 nM Transcreener™ ADP Far Red Tracer. All inhibitors were screened at 10 µM in 1% DMSO in the kinase reaction. The Transcreener™ KINASE Assay is tolerant to DMSO, DMF, ethanol, and acetonitrile at 20%, 6%, 16%, and 10%, respectively. For additional tolerance data, please see the BellBrook Labs website. Tecan Ultra settings and filters: Ex612nm (10 nm bandwidth), Em670nm (25 nm bandwidth), 10 flashes, 30°C. The free tracer reference was set to 20 mP, and buffer (containing Ab) was used as a blank for both the sample and reference wells. Assays were performed in black Corning® plates, catalog # 3676, (384-well, round bottom, low volume, polystyrene, non-binding surface plates), or for higher volumes, in black Corning® plates, catalog #3654 (384-well, flat-bottom plates).

Figure 2. Transcreener™ Monoclonal Antibody: High Selectivity for ADP





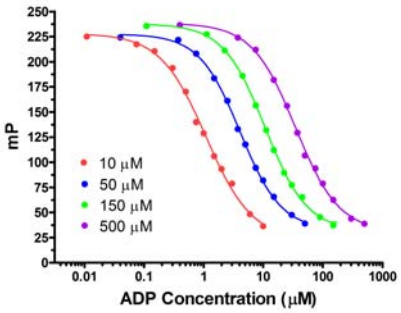
The Transcreener™ monoclonal antibody is a novel anti-ADP antibody that can distinguish between a single phosphate group. Antibody/Tracer complex was mixed with increasing concentrations of ATP, ADP, or AMP to generate the competition curves. The anti-ADP mAb is 100-fold and 7000-fold more selective for ADP, relative to ATP or AMP. The EC_{50} values for ADP, ATP, and AMP are 0.25 μM, 27.8 μM, and 1800 μM, respectively.

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

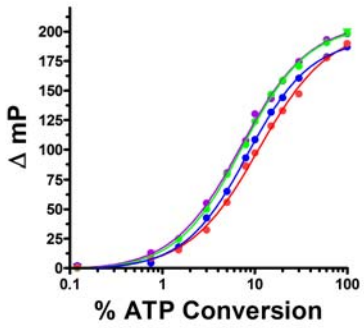
% Conversion	mP Shift	Z'
2	28	-0.06
4	49	0.31
6	64	0.46
8	81	0.58
10	89	0.61
15	115	0.75
20	130	0.77
30	153	0.76
40	164	0.81
50	170	0.75
60	180	0.85
80	187	0.81
100	192	0.85

The Transcreener™ KINASE detection module is robust at low % 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 0.58 to 0.85 were observed over the 8-100 % ATP conversion range.

Figure 3. Transcreener™ KINASE Assay: Flexibility for a Wide Range of ATP Concentrations



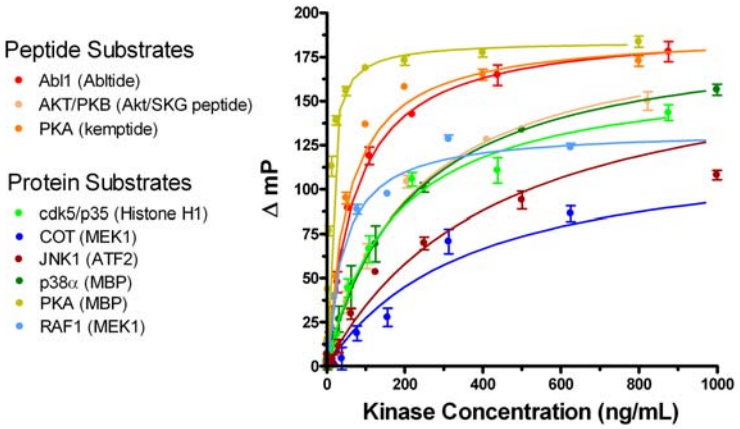
A.



B.

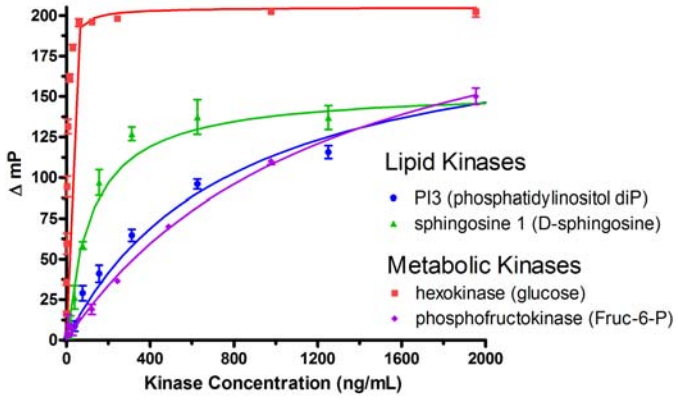
A. ADP Standard Curves at various starting ATP concentrations. To mimic ADP generated during a kinase reaction, standard curves optimized for 10 μM, 50 μM, 150 μM, and 500 μM ATP were prepared by keeping the adenosine concentration constant. The IC_{50} values for the 10 μM, 50 μM, 150 μM, and 500 μM ATP-ADP standard curves are 1.1 μM, 4.1 μM, 10.8 μM, and 34.9 μM ADP, respectively. **B. Excellent signal is maintained over a range of ATP concentrations.** mP shifts greater than 130 are observed at 20% ATP conversion for reactions containing 10-500 μM ATP (starting concentration).

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



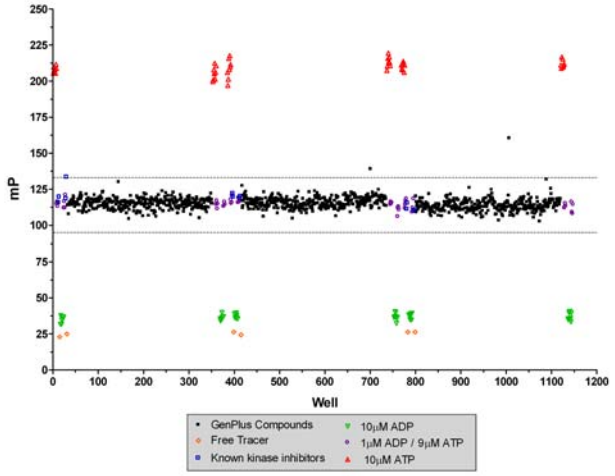
A single set of reagents can be used across kinase families using either peptide or protein substrates. Either Tyr or Ser/Thr kinases were serially diluted two-fold in buffer with either peptide or protein substrates. After the one hour kinase reaction, an equal volume of the Transcreener™ ADP detection mix was added. A subset of relevant kinase targets were tested with a corresponding substrate (in parenthesis) to show the utility of the Transcreener™ KINASE Assay: Abl1 (Abltide), AKT/PKB (AKT/SKG peptide), cdk5/p35 (Histone H1), COT (MEK1), JNK1 (ATF2 aa 19-96), p38 alpha (MBP), PKA (kemptide or MBP), and RAF (MEK1). Kinase reaction buffers were not optimized.

Figure 5. Transcreener™ KINASE Assay Expanded to Lipid and Metabolic Kinases



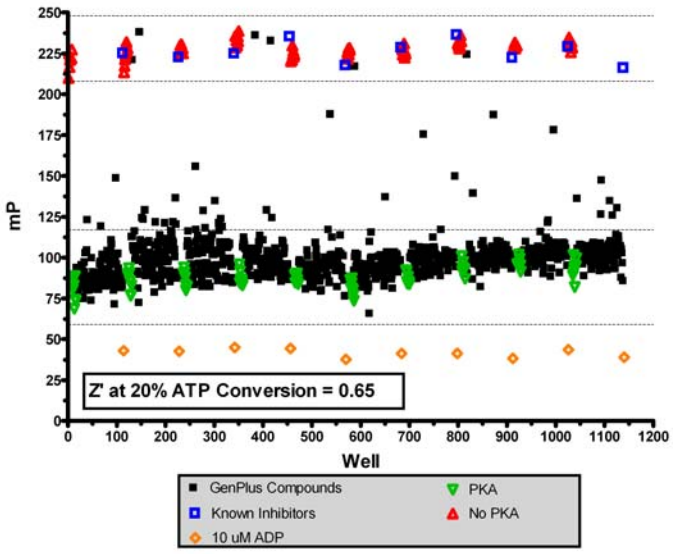
Detecting the invariant product (ADP) enables further expansion of the Transcreener™ KINASE platform to enzymes that phosphorylate lipids or small molecules. Either lipid or metabolic kinases were serially diluted two-fold in buffer containing either lipid or small molecule substrates. The kinases tested with the corresponding substrate (in parenthesis) were PI3 kinase (phosphatidyl inositol diphosphate), sphingosine kinase 1 (D-sphingosine), hexokinase (glucose), and phosphofructokinase (fructose-6-phosphate).).

Figure 6. Compound Interference is Insignificant with the Far Red Transcreener™ KINASE Assay



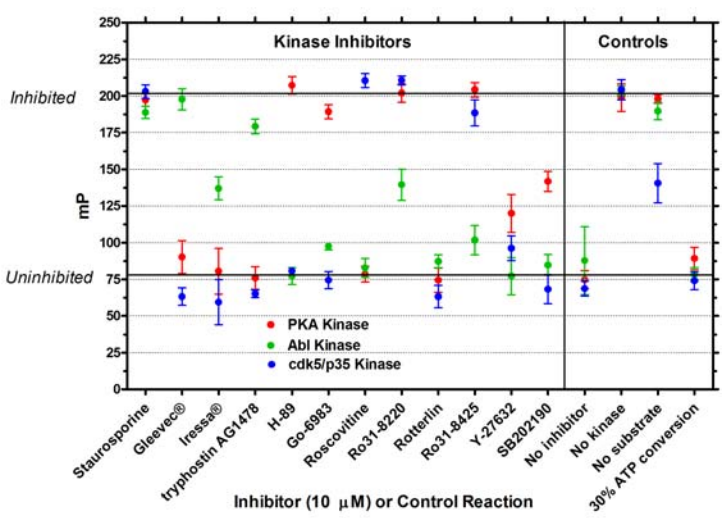
Compounds in the Gen-Plus library are compatible with ADP detection in the Transcreener™ KINASE Assay. Library compounds at 10 µM (1% DMSO) were incubated with 1 µM ADP and 9 µM ATP in the kinase reaction buffer to mimic 10% ATP conversion. An equal volume of the Transcreener™ KINASE Stop and Detect Mix was then added to each well. Of the 972 compounds (960 Gen-Plus compounds + 12 known kinase inhibitors), only 3 generated mP values outside the 3SD of the mean for the 10% ATP conversion control. 100% ATP conversion = kinase buffer containing 10 µM ADP. 0% ATP conversion= kinase buffer containing 10 µM ATP.

Figure 7. PKA Inhibitors Identified in Gen-Plus Compound Library



The Gen-Plus library (960 compounds) and five known kinase inhibitors were screened for PKA inhibition. Test comounds (10 µM in 1% DMSO) were incubated with 20 ng/mL PKA, 50 µM Kemptide, and 10 µM ATP in the standard kinase reaction buffer for one hour at 25 °C. An equal volume of the antibody/tracer detection mix was then added to each well. All compounds were tested in triplicate on the same plate. To calculate the amount of ADP produced during the reaction, twelve point standard curves were prepared on each plate (n=3) (data not shown). Eight control reactions with PKA (20% ATP conversion, n=3) and without PKA(0% ATP conversion, n=3) were included on each plate. To represent 100% ATP conversion, 10 µM ADP was added to the kinase buffer (n=3). All points represent mean values and the dotted lines represent 3 SD of the mean for the control reactions. Known kinase inhibitors include Staurosporine, H-89, GO-6983, Ro-31-8425, and Ro 31-8220.

Figure 8. Transcreener™ KINASE Assay: Accurate Inhibitor Pharmacology



Profiling known inhibitors with PKA, cdk5/p35, and Abl 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. Gleevac® and Tryphostin AG1478 specifically inhibited Abl, whereas H-89 and Go-6983 were selective towards PKA. Roscovitine only inhibited CDK5/p35. As evidenced by a decrease in the mP value, ATP was intrinsically hydrolyzed by CDK5/p35 in the absence of Histone H1.

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 (3 µM to > 250 µM).

3. The Transcreener™ KINASE Assay is sensitive and robust yielding Z' > 0.5 at 8% and greater ATP conversion.

4. A single set of reagents can be used to assay protein, lipid, or metabolic kinases with diverse acceptor substrates.

5. The far red Transcreener™ KINASE Assay is robust; >99.7% of compounds in the Gen-Plus library did not interfere with ADP Detection.

6. At only 20% ATP conversion, the Transcreener™ KINASE Assay successfully identified PKA inhibitors in the Gen-Plus compound library (Z' = 0.65).

7. 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™ HTS 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.

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