ADP Detection Assay for Diverse Kinases and ATPases

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Introduction

The Transcreener™ KINASE Assay is a homogeneous, competitive fluorescence polarization HTS assay that measures ADP, the invariant reaction product of all kinase reactions. The assay consists of an anti-ADP antibody and a far red ADP-Alexa Fluor® 633 tracer which are used to measure product formation from any ADP-producing enzyme including protein kinases, lipid kinases, metabolic kinases, and ATPases. Likewise, the assay can be easily optimized to accommodate a wide range of ATP concentrations. Excellent Z' are obtained at 10% ATP conversions. In this study the Transcreener™ KINASE Assay was used to screen the Gen-Plus compound library against PKA. Use of a far red tracer significantly reduces compound interference. This single powerful assay platform can be used to profile kinases, ATPases, substrates, and inhibitors leading to reduced development costs, increased assay flexibility and accelerated drug discovery.

Figure 1. Transcreener™ KINASE Assay Measures ADP

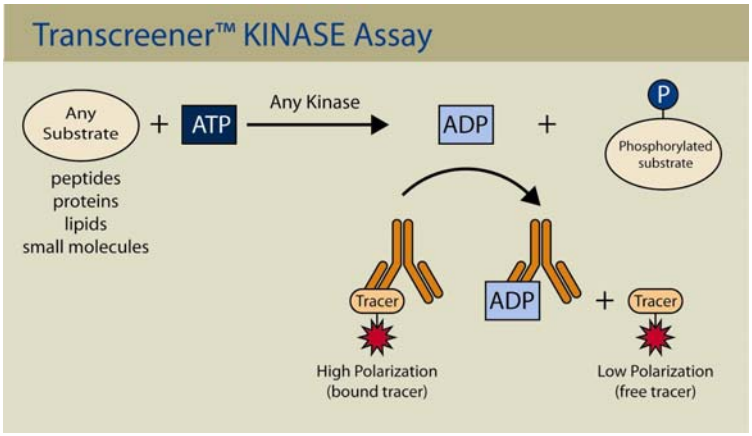
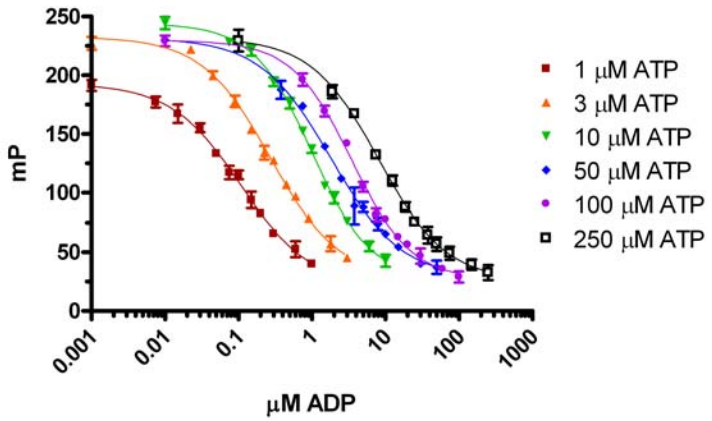


Figure 2. Transcreener™ KINASE Assay Accomodates a Wide Range of [ATP] Yields Excellent Z' at 10% ATP Conversion.

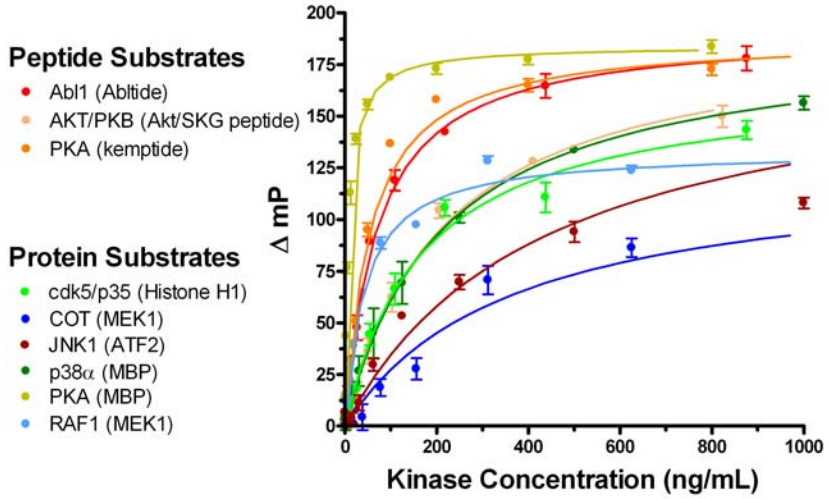


ADP/ATP Standard Curves at various starting ATP concentrations. To mimic ADP generated during a kinase reaction, standard curves optimized for 1, 3, 10, 50, 100, and 250 µM ATP were prepared by keeping the adenosine concentration constant. The IC_{50} values for the ATP/ADP standard curves are 0.1, 0.27, 1.0, 1.8, 3.5, and 8.0 µM ADP, respectively.

µM ATP (kinase rxn)	[Ab] final	LLD (nM)	Δ mP at 10% Conversion	Z' at 10% Conversion
1	1.0	10	77	0.51
3	2.5	30	99	0.65
10	10	90	114	0.61
50	25	200	140	0.76
100	50	250	148	0.78
250	80	500	153	0.73

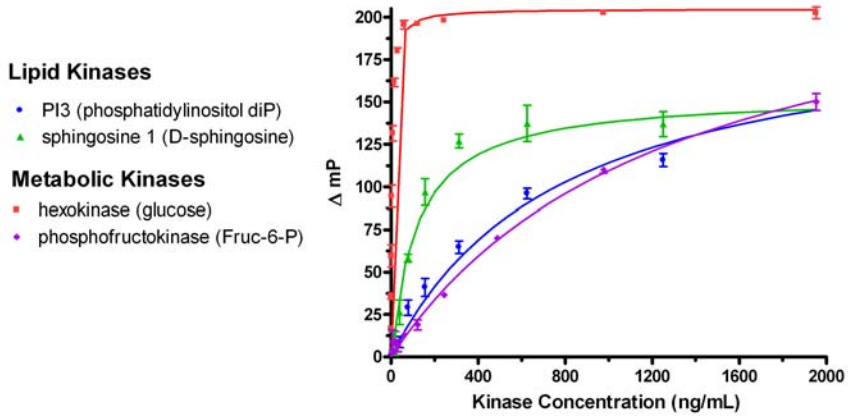
Amount of antibody required to achieve the greatest mp shift and Z' > 0.5 at 10% ATP conversion for various starting ATP.

Figure 3. Transcreener™ KINASE Assay: Any Kinase, Any Substrate



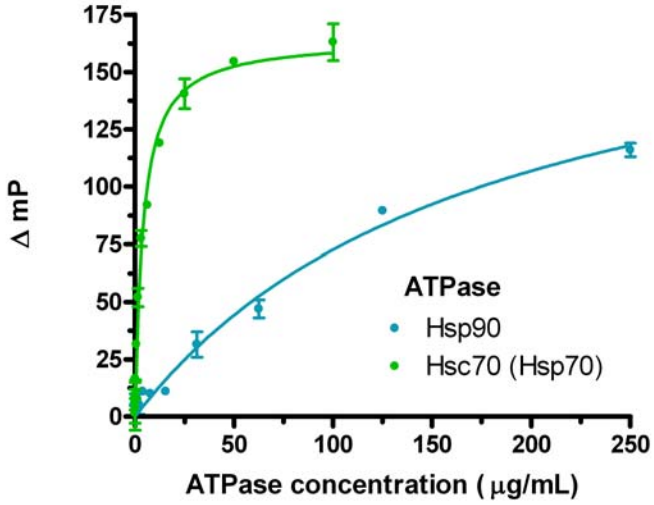
Tyr or Ser/Thr kinases were serially diluted two-fold in reaction buffer with either peptide or protein substrates (in parenthesis). Kinase reactions were incubated for one hour and then ADP was measured after addition of an equal volume of ADP detection mix. Kinase reaction buffers were not optimized. A single set of reagents was used with all kinases tested.

Figure 4. Transcreener™ KINASE Assay Expanded to Lipid and Metabolic Kinases



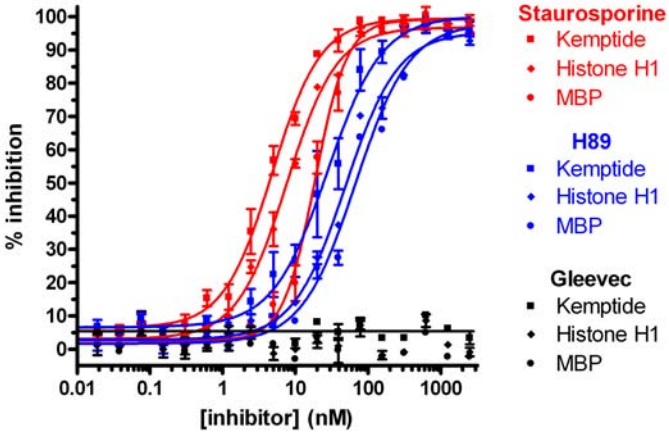
The Transcreener™ KINASE Assay measures ADP-production and can be used to assay the entire kinome including non-protein kinases that phosphorylate lipid and carbohydrate substrates. Enzymes were serially diluted in buffer containing appropriate substrates (in parentheses). After one hour incubation with ATP, ADP was measured in the reaction by adding an equal volume of ADP Detection Mix.

Figure 5. Transcreener™ KINASE Assay Detects Activity of ATPases.



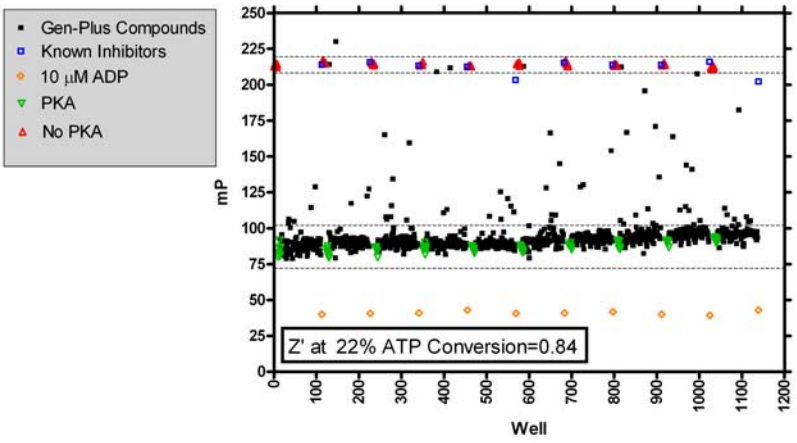
The Transcreener™ KINASE Assay was also be used to measure ADP produced by an important class of ATPases, namely heat shock proteins. Hsp90 (human heat shock protein 90) and Hsc70 (constitutive bovine Hsp70) were incubated with 50 μM ATP for 37 °C for 3 hours.

Figure 6. PKA Inhibitor Potency against Peptide and Protein Substrates is Confirmed.



The IC_{50} values for inhibition by Staurosporine were 4.8 nM, 7.6 nM, and 18.7 nM using Kemptide, Histone H1, and MBP substrates, respectively. The IC_{50} values for inhibition by H89 were 27.6 nM, 49.0 nM, and 67.1 nM with Kemptide, Histone H1, and MBP substrates, respectively. As expected, Gleevec did not inhibit PKA activity. PKA kinase assays were performed in kinase reaction buffer at 10 μM ATP, with 50 μM (Kemptide), 5 μM (Histone H1), or 20 μM (MBP) substrate. For Kemptide (15 ng/mL PKA), Histone H1 (30 ng/mL PKA), and MBP (600 ng/mL PKA) produced 17%, 11%, and 16% ATP conversion, respectively.

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



The Gen-Plus library (960 compounds) and five known kinase inhibitors were screened in triplicate for PKA inhibition. Test compounds (10 μM in 1% DMSO) were incubated with 12 ng/mL PKA, 50 μM Kemptide, and 10 μM ATP in the standard kinase reaction buffer. Twenty-four control reactions with PKA (22% ATP conversion) and without PKA (0% ATP conversion) were included on each plate. All points represent mean values and the dotted lines represent 3 SD of the mean for the control reactions. Known kinase inhibitors (n=2) include Staurosporine, H-89, GO-6983, Ro-31-8425, and Ro-31-8220. Insignificant compound interference (0.3%) with the far-red tracer was confirmed in a control compound screen. (Data not shown.)

Materials and Methods

In general the Transcreener™ KINASE Assay was performed as a two-part, endpoint assay (a 10 µL enzyme reaction followed by the addition of 10 µL ADP Detection Mixture). Kinase reaction conditions: 50 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 2 mM EGTA, 1-500 µM ATP, 50 µM peptide substrate, 0.3-10 µM protein substrates (Histone H1, MEK1, or MBP), 20-50 µM lipid substrate, and 100 µM glucose or fructose-6-phosphate substrates. Unless noted, all kinase reactions were incubated for one hour at 25°C. ATPase reaction conditions: 50 mM HEPES (pH 7.5), 2 mM $MgCl_2$, 2 mM EGTA, 20 mM KCl, 0.01% Brij-35, 50 µM ATP. The reactions were performed at 37°C for 3 hours. Transcreener™ ADP Detection Mixture: 50 mM HEPES (pH 7.5), 400 mM NaCl, 20 mM EDTA, 0.01% Brij-35, 5-260 µg/mL Transcreener™ ADP Ab, and 4 nM Transcreener™ ADP Far-Red Tracer. 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). Tecan Ultra settings and filters: Ex612(10 nm bandwidth), Em670(25 nm bandwidth), 10 flashes, 30°C.

Conclusions:

1. The Transcreener™ KINASE Assay can be optimized to accommodate a wide range of ATP concentrations (1 µM to > 250 µM).

2 $Z' > 0.5$ at 10% ATP conversion for the entire range of starting [ATP].

3. The Transcreener™ KINASE Assay measures product formation from any ADP producing enzyme including protein, lipid, or metabolic kinases, and even ATPases.

4. Staurosporine and H89 PKA inhibitor potency is confirmed against peptide and protein substrates.

5. At 22% ATP conversion, the Transcreener™ KINASE Assay successfully identified PKA inhibitors in the Gen-Plus compound library ($Z' = 0.84$).

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

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