Targeting Low Activity ATP-utilizing Enzymes with the Improved Transcreener® ADP² FP Assay

Karen Kleman-Leyer[1], Tony A. Klink[1], Andrew L. Kopp[1], Tracy J. Worzella[1], Cori A. Pinchard[1], and Robert G. Lowery[1]
[1]BellBrook Labs, Madison, WI, USA

Overview

BellBrook's Transcreener® platform is the only HTS assay method that allows direct detection of ADP formed by kinases and other ATP-utilizing enzymes. A highly selective antibody that recognizes ADP in the presence of excess ATP allows robust measurements of enzyme initial velocity without the use of coupling enzymes (e.g., luciferase), thus providing a simpler method and eliminating a potential source of interference. The first generation Transcreener ADP Assay has been successfully deployed in HTS laboratories since 2006, where it has been used to generate more than 4M data points for diverse ATP-utilizing drug targets, including protein, carbohydrate, and lipid kinases as wells as emerging targets such as heat shock proteins, carboxylases and other ATPases. Here we describe the recent development of an improved monoclonal antibody with 20-fold higher affinity for ADP (LLD = 1 nM ADP in the presence of 99 nM ATP) and similar selectivity (>100-fold) vs. ATP. Competitive binding experiments indicate higher affinities for other nucleotide diphosphates in addition to ADP, including GDP and UDP. The new antibody also yields a higher polarization shift when in complex with tracer, thus increasing the assay window. These combined properties improve data quality, as reflected in higher Z′ values, over a broad range of conditions and extend the utility of the Transcreener ADP² FP Assay to applications requiring lower amounts of ATP. For example, the improved assay enables robust detection of enzyme initial velocity (Z′ > 0.7 at ≤ 20 % substrate consumption) with initial ATP concentrations spanning four logarithms (0.1–1,000 µM ATP). The assay reagents - antibody and tracer – are stable for weeks at room temperature and the assay signal shows essentially no change over 24 hours at room temperature. In addition, the linear relationship between the optimal antibody concentration (EC_{85}) and ATP concentration is highly consistent in replicate experiments and using multiple batches of antibody. This allows the user to calculate the optimal amount of antibody to use for assays run at any ATP concentration between 100 nM and 1 mM, eliminating the need for pilot experiments.

The Transcreener® ADP² FP Assay

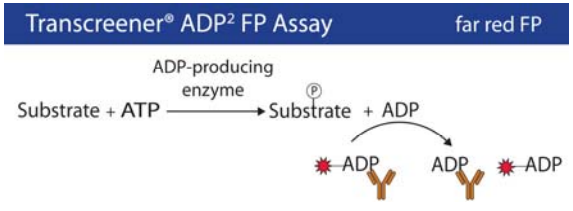


Figure 1. The Transcreener ADP² Assay enables direct detection of ADP using a competitive fluorescence polarization immunoassay. The assay relies on a highly selective antibody that binds ADP with more than 100-fold higher affinity than ATP. The Transcreener ADP Detection Mixture comprises an ADP Alexa633 Tracer bound to the ADP² antibody. The tracer is displaced by enzymatically produced ADP, resulting in decreased polarization. The far red tracer minimizes interference from fluorescent compounds and light scattering.

The ADP² Antibody has Increased NDP Sensitivity

Nucleotide	IC_{50} (nM) ADP Ab	IC_{50} (nM) ADP² Ab	NDP Affinity Improvement
ADP	180	7.9	23x
ATP	17,300	915	
ATP/ADP	96	92	
GDP	50	10.7	12x
GTP	1,500	1,400	
GTP/GDP	30	131	
UDP	ND	214	ND
UDP-Glc	ND	744,000	
UDP-Glc/UDP	ND	3,476	

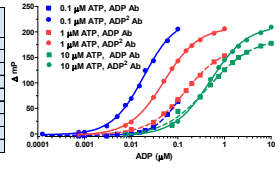


Table 1 and Figure 2. The new ADP antibody increases sensitivity of ADP detection more than 20-fold and enables detection of other NDPs. Nucleotides were titrated in the presence of ADP antibodies and an Alexa-Fluor® 633-ADP tracer in 384-well microplates. Fluorescence polarization measurements were made on a Tecan Safire²™ multi-mode plate reader. Competition curves were used to generate IC_{50} values. Reaction conditions were optimized separately for each antibody. The functional effects of increased affinity can be seen in the standard curves for ATP-ADP conversion at the right. At 0.1 and 1 µM initial ATP concentrations, the ADP² antibody enables detection of much lower levels of conversion; at initial ATP concentrations of 10 µM and higher, the two antibodies exhibit similar sensitivity, but the increased polarization shift is still observed for the ADP² antibody.

Reagent and Signal Stability for HTS

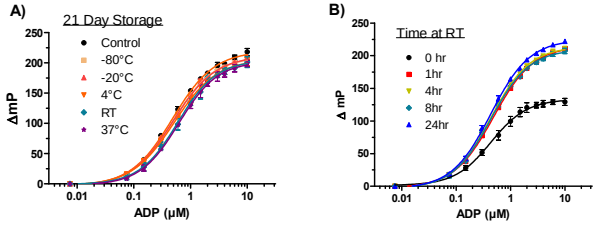


Figure 3. Reagent and Signal Stability. (A) Signal stability measured using a 10 µM ATP/ADP standard curve. The 10 µM ATP/ADP standard curve was prepared in the presence of ADP² antibody and tracer in 384 well plates and the FP signal was recorded at the indicated times. Error bars represent standard deviations of the mean (n = 24). B) Reagent stability at RT. ADP² antibody and tracer were combined and stored for 21 days at the designated temperatures prior to adding to freshly prepared ATP/ADP standards. The Day 0 control standard curve was prepared with fresh ADP Detection Mixture. Error bars represent standard deviations of the mean (n = 4). $\Delta mP = mP_{10\mu M\ ATP} - mP_{ATP/ADP}$.

Use 0.1 to 1,000 µM ATP with Simplified EZ Protocol

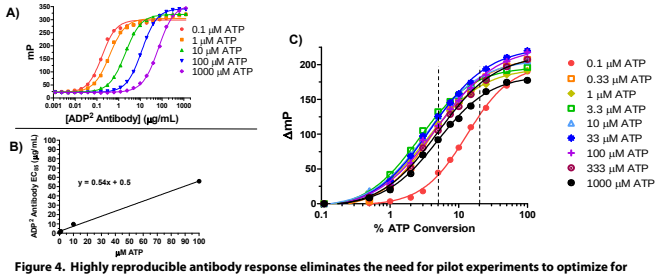


Figure 4. Highly reproducible antibody response eliminates the need for pilot experiments to optimize for different ATP concentrations. (A) For an optimal assay window in detecting enzyme initial velocity (ca. 5-20% ATP conversion), BellBrook recommends using antibody at 85% of saturation binding (EC_{85}). This value increases as ATP is increased because of cross reaction with ATP. (B) The relationship between EC_{85} and ATP is linear and very stable . (C) Using the EC_{85} concentrations calculated from the equation for this line (in the Technical Manual) allows turnkey detection of initial velocity ADP levels at any initial ATP concentration.

More Sensitive than ATP Depletion Methods

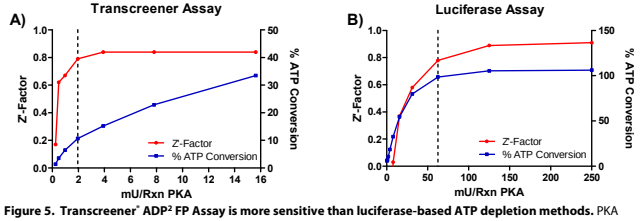


Figure 5. Transcreener® ADP² FP Assay is more sensitive than luciferase-based ATP depletion methods. PKA enzyme reactions were performed in 384-well plates in a 10 µL reaction volume with 10 µM ATP and 50 µM Kemptide substrate for one hour at room temperature. The enzyme buffer recommended by the Luciferase Assay vendor was used for both assays and fluorescence polarization and luminescence were measured on a BMG LABTECH PHERAstar *Plus* plate reader. (A) Transcreener: an equal volume of ADP Detection Mixture was added to stop the reaction, followed by a 10 minute equilibration before reading. The equilibration period was shortened from the usual one hour period to be consistent with the Luciferase Assay protocol. (B) Luciferase: an equal volume of Luciferase Assay reagent was added to the reaction, followed by a 10 minute equilibration before reading. As shown by the dashed lines, 30-fold less enzyme is needed to achieve a Z′ > 0.7 with the Transcreener assay with lower % ATP conversion (A) versus the ATP depletion assay (B).

ADP Detection From Enzymes With Low ATP K_m

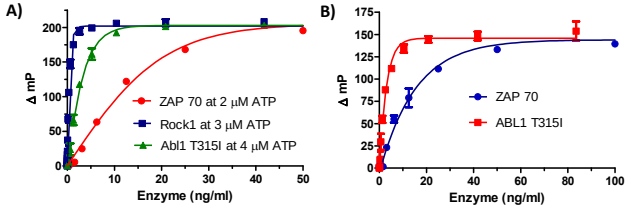
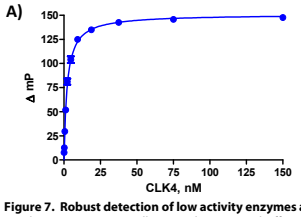


Figure 6. Enhanced ADP detection translates to robust detection of enzymes with low ATP requirements. Dose response curves for kinases in the presence of (A) ATP at the corresponding K_m concentration, or (B) 0.1 µM. Experiments were performed in Corning Low Volume 384-Well Plates (# 3676). Fluorescence polarization was read in a PerkinElmer EnVision® multilabel reader. Free tracer was set to 20 mP, and buffer containing antibody was used for blank correction. Kinase reactions were run in CK buffer (50 mM HEPES (pH 7.5), 0.01% Brij-35, 2 mM EGTA, 4 mM $MgCl_2$) in 10 µL final volume. Final read volume after addition of Stop & Detect Mix was 20 µL. Stop and Detect Mix contained (final assay concentrations) 2 nM Alexa 633-ADP and ADP² Ab at concentrations appropriate for the starting [ATP].

ADP Detection From Low Activity Enzymes



B)

Clk4, nM	Z′	Δ mP	µM ADP Produced	% Conversion
0.1	-0.58	8	< 0.005	< 0.5
0.3	-0.06	13	< 0.005	< 0.5
0.6	0.57	30	0.008	0.8
1.2	0.79	52	0.018	1.8
2.3	0.82	82	0.037	3.7
4.7	0.85	104	0.06	6
9.4	0.92	125	0.097	9.7
18.8	0.93	135	0.134	13.4
37.5	0.92	143	0.213	21.3
75.0	0.93	146	> 1	> 100
150.0	0.94	148	> 1	> 100

Figure 7. Robust detection of low activity enzymes at low ATP (1 µM). (A) CLK4 (specific activity 26 nmole/min/mg) was serially titrated in enzyme buffer and reactions were started with the addition of ATP and RS peptide substrate and incubated for one hour. The final concentration of reaction components in the 10 µL reaction volume included 1X enzyme buffer (25 mM Tris (pH 7.5), 10 mM $MgCl_2$, 0.5 mM EGTA, 2.5 mM DTT, 0.01% Triton-X 100), 1 µM ATP, and 100 µM RS peptide. 10 µL of Stop & Detect Mix was added to stop the enzyme reaction, the microplate was mixed and allowed to equilibrate for one-hour before reading on a Tecan Safire²™ reader. The final concentration of detection components in the 20 µL reaction was 1 µg/mL ADP² antibody and 2 nM Alexa 633 tracer. (B) Excellent Z′ values are achieved down to an enzyme concentration of 0.6 nM, detecting as little as 0.8 % conversion of ATP to ADP.

Conclusions

- A higher affinity monoclonal antibody that enhances sensitivity for ADP more than 20-fold (IC_{50} = 7.9 nM) has been developed and incorporated into the recently introduced Transcreener ADP² FP Assay.
- The Transcreener ADP² FP Assay enables robust detection (Z′ > 0.7) of kinase velocity using ATP concentrations as low as 100 nM and as high as 1 mM. A consistent linear relationship between ATP concentration and antibody response eliminates the need for pilot experiments to optimize Ab.
- The enhanced sensitivity and flexibility for ATP allows facile screening of enzymes with low ATP requirements (< 5 µM) or low specific activity (CLK4).
- In direct comparisons with luciferase-based ATP depletion methods, the Transcreener ADP² FP Assay enabled robust PKA detection (Z′ > 0.7) using 30-fold less enzyme (2 mU vs. 60 mU) and much lower ATP conversion (10% vs.100%).
- The improved ADP antibody also enables highly selective detection of GDP and UDP, allowing generic detection for GTPases and UDP-sugar dependent glycosyltransferases.

BellBrook Labs 5500 Nobel Drive, Suite 250, Madison, WI 53711
866.313.7881 or 608.443.2400

www.bellbrooklabs.com