Targeting Low Activity ATP-utilizing Enzymes with the Improved Transcreener® ADP 2 FP Assay

Karen Kleman-Leyer1, Tony A. Klink1, Andrew L. Kopp1, Tracy J. Worzella1, Cori A. Pinchard1, and Robert G. Lowery1
1BellBrook 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 ADP-utilizing drug targets, including protein, carbohydrate, and lipid kinases as well as emerging targets such as heat shock proteins, carbonic anhydrase and other ATPases. Here we describe the recent development of an improved monoclonal antibody with 20-fold higher affinity for ADP (Kd < 1 nM ADP in the presence of 99 nM ATP) and similar selectivity (≥ 100-fold for ATP). These improvements enable higher sensitivity, improved specificity, and robustness of kinetic measurements. In addition to ADP, including GDP and UDP. The new antibody also yields a higher signal-to-noise ratio when in complex with tracer, thus increasing the assay’s accuracy. 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 ADP FP Assay in applications requiring lower amounts of ATP. For example, the improved antibody enables robust detection of enzyme initial velocity (≥ 0.7 % substrate consumption) with initial concentrations spanning four logarithms (0.1–1,200 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 (EC50) 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® ADP 2 FP Assay

The Transcreener® ADP 2 FP Assay allows 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 ADP 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

Table 1 and Figure 2. The new ADP antibody increases sensitivity of ADP detection more than 20-fold and enables detection of other NDPs. Selectivity was 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 Safire® multi-mode plate reader. Competition curves were used to generate IC50 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 and other ADP-utilizing enzymes. The new antibody improves the detection of even 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 ADP 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).  Reagent stability at RT: ADP and 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).  ΔmP = mP10μM ATP – mP0.1μM ATP.  (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.

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 (≥ 5-20% ATP conversion), BellBrook recommends using antibody at 85% of saturation binding (EC50). This value increases as ATP level is increased because of cross reaction with ATP.  The relationship between EC50 and ATP is linear and very stable.  (C) Using the EC50 concentrations calculated from the equation for this line (in the Technical Manual) allows turnkey detection of initial velocity ATP levels at any initial ATP concentration.

More Sensitive than ATP Depletion Methods

Figure 5.  Transcreener® ADP 2 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 peptide substrate for one hour at room temperature. The enzyme buffer recommended by the Luciferase Assay vender was used for both assays and fluorescence polarization and luminescence were measured on a B&G LABCHEMPLUSII Multi 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.  Based on the dashed lines, 30- fold less enzyme is needed to achieve a Z’ ≥ 0.7 with the Transcreener assay when using % ATP conversion (A) versus the ATP depletion assay (B).

ADP Detection From Enzymes With Low ATP Km

Figure 6.  Enhanced ADP detection relates to robust detection of enzymes with low ATP requirements.  Dose response curves for kinase in the presence of (A) ATP at the corresponding Km concentration, or (B) 0.1 μM.  Experiments were performed in Corning Low Volume 384-Well Plates (#1670).  Fluorescence polarization was read in a PerkinElmer Envision® multilabel reader.  Free tracer was set to 20 nM and buffer containing antibody was used for blank correction.  Kinase reactions were run at 37°C buffer (50 mM Hepes (pH 7.5), 0.1% Brij-35, 2 μM EDTA, 4 mM MgCl2).  μM ATP final volume.  Final read value after addition of Stop & Detect Mix was 20 μL.  Stop and Detect Mix contained final assay concentrations: 2 mM ATP 633-ADP and ATP-α-bi, which concentrations are appropriate for the starting ATP.

ADP Detection From Low Activity Enzymes

Figure 7.  Robust detection of low activity enzymes at low ATP (1 μM).  (A) CLK1-specific activity 26 mU/mg (10 μg) was serially diluted in enzyme buffer and reactions were started with the addition of 50 μM ADP and 85 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 MgCl2, 0.5 mM EDTA, 2.5 mM DTT, 0.01% Triton-x 100), 1 μM ATP, and 100 μM peptide.  10 μL of Stop & Detect Mix was added to stop the reaction, the microplate was mixed and allowed to equilibrate for one hour before reading on a Tecan Safire™ reader.  The final concentration of detection components in the 250 μL reaction was 1 μM ATP, ADP antibody and 3 μM 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 highly affinity monoclonal antibody that enhances sensitivity for ADP more than 20-fold (EC50 = 7-9 nM) has been developed and incorporated into the recently introduced Transcreener ADP 2 FP Assay.
- The Transcreener ADP 2 FP Assay enables robust detection (Z’ ≥ 0.7) of kinase activity using ATP concentrations as low as 100 nM and as high as 1 M.  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 ADP 2 FP Assay enabled robust PKA detection (Z’ = 0.7) using 30-fold less enzyme (2 μL vs. 60 μL) and much lower ATP conversion (10% vs. 100%).
- The improved ADP antibody also enables highly sensitive detection of GTPases and UDP-sugar dependent glycosyltransferases.

©2009 BellBrook Labs. All rights reserved.
Transcreener is a registered trademark of BellBrook Labs.
Transcreener® is a registered trademark of Molecular Probes, Inc (Invitrogen).
BellBrook Labs   5500 Nobel Drive, Suite 250, Madison, WI 53711 866.313.7881 or 608.443.2400
www.bellbrooklabs.com