Generate Improved Fluorescence Polarization Screening Results on PerkinElmer's EnVision™ Microplate Reader Through the Use of the Transcreener® ADP² FP Assay

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Overview

BellBrook Labs has developed a Transcreener® Instrumentation Validation Program for plate reading instrumentation. Instruments were evaluated by running a 384-well plate containing a 10 µM ADP/ATP standard curve on the microplate reader using variable flashes. This curve mimics the conversion of ATP to ADP, as seen in an enzyme reaction. While all microplate readers yielded Z′ values > 0.5 at 10% ATP conversion levels, PerkinElmer's EnVision™ was not able to meet the higher standards required for validation which include a Z′ ≥ 0.7, and a Δ mP ≥ 95 at 10% conversion of 10 µM ATP. BellBrook recently developed the Transcreener ADP² FP Assay. Through the incorporation of the more sensitive ADP² Antibody, improved Δ mP values are seen at less than or equal to 10% ATP conversion across a broader range of initial ATP concentrations (0.1 µM to 1,000 µM). When validation experiments were repeated, the EnVision showed improved results using the ADP² FP Assay and met the criteria of the Validation Program. Standard curve comparison data, run at 10 µM ATP on the EnVision, shows the ability of the assay to deliver higher quality data. Enzyme data with ROCK1 kinase demonstrate excellent results with the ADP² FP Assay at low ATP concentrations, as well as with low % ATP conversion levels, which are more inline with initial velocity conditions.

Introduction

BellBrook Labs has developed a universal assay technology called Transcreener® HTS Assay Platform that enables seamless incorporation of hundreds of new drug targets into HTS. The Transcreener HTS platform relies on a proprietary fluorescence detection method for group transfer enzymes that enables an entire family of enzymes to be screened using the same detection reagents. The Transcreener ADP² FP Assay is a universal, second generation far red fluorescence polarization assay that detects the ADP nucleotide produced in most ATP-utilizing enzyme reactions. The increased sensitivity of the new ADP² antibody allows for detection of ADP at less than 10% ATP consumption, or within initial velocity enzyme reaction rates, and at broader ATP concentrations ranging from 0.1 µM to 1,000 µM.

The ability to demonstrate the capabilities of a screening technology on existing laboratory instrumentation is essential in today's market. BellBrook Labs set out to do this through its Transcreener Instrumentation Validation Program. The Program is designed to test the compatibility of the Transcreener assay on commonly used detection instrumentation, such as the PerkinElmer EnVision™ HTS reader. We evaluated filter sets and number of flashes per well using a 10 µM ADP/ATP standard curve. From the curve we determined the Z′-factor and mP shift at 10% ATP conversion at the different settings. The EnVision showed excellent results with the ADP² FP Assay and met all the criteria of the Validation Program. The attributes of the new Transcreener Assay were demonstrated on the EnVision by performing ROCK1 kinase titrations at 3 µM ATP, the ATP K_m for ROCK1. Validation data and studies with ROCK1 show that the Transcreener Assay and EnVision are a validated combination for researchers looking to expand their high throughput screening campaigns to include low activity enzymes, or those possessing low ATP K_m.

The Transcreener® ADP² FP Assay

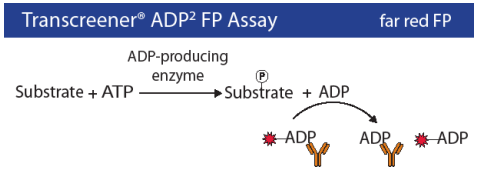

Figure 1. The Transcreener ADP² Assay. The Transcreener ADP² Assay was developed to follow the progress of any enzyme that produces ADP. The Transcreener ADP Detection Mixture comprises an ADP Alexa633 Tracer bound to an ADP² antibody. The tracer is displaced by ADP, the invariant product generated during an enzyme reaction. The displaced tracer freely rotates in solution leading to a decrease in fluorescence polarization. Therefore, ADP production is proportional to a decrease in polarization. The far red tracer minimizes interference from fluorescent compounds and light scattering.

The PerkinElmer EnVision™ HTS Multilabel Plate Reader

Figure 2. The EnVision HTS Multilabel Plate Reader. The EnVision is capable of reading 96, 384, and 1536-well assay plates. Filters and dichroic modules are interchangeable and easy to install. The simultaneous dual-detection capabilities of the reader enable fast read times. For far red FP, we used the following: optimized Cy5 FP Dual Emission Label, 620/40 nm excitation filter, 688/45 nm emission filter, and D658/fp688 dual mirror.

Validation at 10% ATP Conversion With ADP Assay

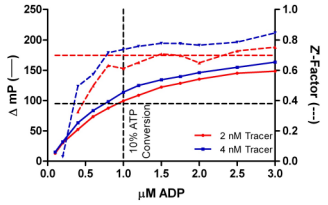

Figure 3. EnVision passes validation with ADP assay using modified protocol. The EnVision did not meet criteria of the Instrument Validation Program with the original Transcreener ADP FP Assay using standard conditions. The validation criteria include: Z′ ≥ 0.7 at 10% conversion at 10 µM ATP, a Δ mP shift ≥ 95 mP at 10% conversion and read time ≤ 5 minutes. Z′ = 0.6 with Δ mP shift of 95 was achieved with 2 nM ADP Alexa633 tracer at 10% ATP conversion with 150 flashes. Increasing tracer concentration to 4 nM improved results, resulting in a Z′ = 0.74 with Δ mP shift of 114 at 10% ATP conversion with 150 flashes and read time of 3 minutes, 1 second.

Note: The ADP FP Assay has since been replaced with the ADP² FP Assay.

Validation at 10% ATP Conversion With ADP² Assay

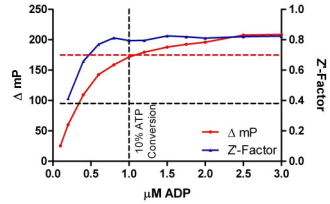

Figure 4. EnVision passes validation with ADP² assay without modifications to protocol. The validation criteria include: Z′ ≥ 0.7 at 10% conversion of 10 µM ATP, a Δ mP shift ≥ 95 mP at 10% conversion and read time ≤ 5 minutes. Z′ = 0.79 with Δ mP shift of 172 was achieved with 2 nM tracer at 10% ATP conversion with 30 flashes and read time of 1 minute, 20 seconds.

ADP² Antibody Optimization For 3 µM ATP

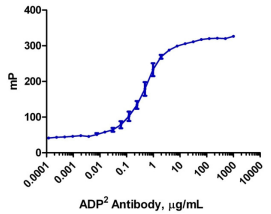

Figure 5. Optimizing ADP² antibody concentration for ROCK1 kinase reaction at 3 µM ATP. The ADP² antibody has a finite selectivity for the nucleotide diphosphate (ADP) vs. the nucleotide triphosphate (ATP). The concentration of antibody determines the total assay window and the ADP detection range. Therefore, antibody must be titrated in the reaction system and ATP concentration desired in the assay. ADP² antibody was titrated as shown using reaction components for ROCK1 kinase. The final 20 µL volume consisted of 2 nM ADP Alexa633 tracer, 0.5X Stop & Detect Buffer B, 0.5X enzyme reaction mixture (25 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.005% Brij-35, 1.5 µM ATP, 76 µM S6 kinase substrate), and ADP² antibody. The EC_{85} antibody concentration was 2.9 µg/mL. $EC_{85} = ((85/(100-85))^{1/hillslope})*EC_{50}$.

ROCK1 Kinase Titration

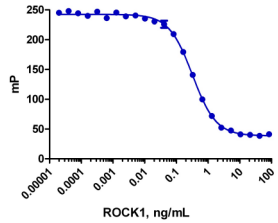

Figure 6. ROCK1 kinase titration. ROCK1 serial titration (1:2) was performed in a 10 µL reaction volume at the ATP K_m (3 µM ATP) with 152 µM S6 kinase substrate for one hour at room temperature. An equal volume of Detection Mixture was added and equilibrated for one hour before reading. The final 20 µL assay volume containing the Detection Mixture consisted of 2 nM ADP Alexa633 tracer, 0.5X Stop & Detect Buffer B, 2.9 µg/mL ADP² antibody, and 0.5X enzyme reaction mixture (25 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM EGTA, 0.5% DMSO, 0.005% Brij-35, 1.5 µM ATP and 76 µM substrate). EC_{50} was 0.32 ng/mL enzyme. 10% ATP conversion was achieved with 1.4 ng/mL of enzyme.

Conclusions

- For optimal performance of the Transcreener ADP² Assay on the EnVision HTS reader, we recommend the following: optimized Cy5 FP Dual Emission Label, 620/40 nm excitation filter, 688/45 nm emission filter, and D658/fp688 dual mirror.
- The EnVision passes BellBrook Labs' Validation Program with the ADP² Assay under the following conditions: 30 flashes, Δ mP = 172 at 10% ATP conversion. These settings yielded a Z′ > 0.7 in 1 minute, 20 seconds.
- Enzymes with low ATP K_m, such as ROCK1 kinase, can be easily optimized with Transcreener within initial rate conditions (≤ 10% ATP conversion).

Visit www.bellbrooklabs.com for a detailed Application Note describing the validation of the EnVision with the Transcreener ADP² FP Assay.

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