Volume Scaling of the Transcreener® ADP² FP Assay on the BioTek Synergy™ 2 and 4 Multi-Mode Microplate Readers Allows For Easy Transition From Assay Development to High-Throughput Screening

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Overview

We present data showing the validation of BellBrook Labs' Transcreener® ADP² FP Assay on the BioTek Synergy™ 2 and Synergy™ 4 plate readers. The Transcreener ADP² Assay is a universal, second generation far red fluorescence polarization assay that detects the ADP nucleotide by-product from any ATP-utilizing enzyme reaction. The assay is amenable to volume scaling, as shown by data generated in 96-well and 384-well microplate formats. Data generated with a standard curve in both microplate formats resulted in Z´ values ≥ 0.6. Further, the 96-well assay using the standard PMT and the 384-well assay using the red-shifted PMT met BellBrook Labs' Instrument Validation Program criteria, which include Z´ > 0.7 with Δ mP shift ≥ 95 at 10% conversion of 10 µM ATP to ADP. Further enzyme studies demonstrate that 96- and 384-well plate formats yield excellent Z´-factor results with Protein Kinase A (PKA) at low % ATP conversion. Known PKA inhibitors show comparable potency in both 96- and 384-well plate formats, and between different Synergy plate readers. Although the standard PMT Synergy does not meet validation criteria in 384-well plate format, comparable inhibitor potency results are obtained between the standard and red-shifted PMT instruments.

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 at initial velocity enzyme reaction rates, and at broader ATP concentrations ranging from 0.1 µM to 1,000 µM.

A critical factor in achieving success with an assay is to ensure that the detection instrumentation is compatible with the reagents being used. Assay reagents must also demonstrate scalability from larger volume to smaller volume wells to accommodate higher throughput processing for the transition from assay development to screening. We present data showing the validation of BellBrook Labs' Transcreener ADP² FP Assay on the BioTek Synergy™ 2 and Synergy™ 4 plate readers. We evaluated different filter sets and number of flashes per well using a 10 µM ADP/ATP standard curve in both 96- and 384-well format. From the curve we determined Z´-factor and mP shift at 10% ATP conversion at different settings.

Attributes of the new Transcreener Assay were demonstrated in both well formats by performing PKA titrations, Z´-factor determinations, and inhibitor potency studies. Results indicate that researchers can have confidence in both reagent and reader performance regardless of which well format or detection instrumentation they are using.

The Transcreener® ADP² FP Assay

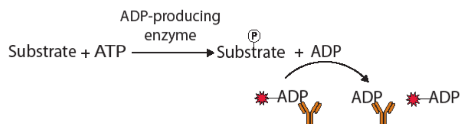


Figure 1. The Transcreener ADP² Assay. The Transcreener ADP² Assay was developed to follow the progress of any enzyme that produces ADP. The Transcreener ADP Detection Mixture comprises an ADP Alexa633 Tracer bound to an ADP² antibody. The tracer is displaced by ADP, the invariant product generated during 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 BioTek™ Synergy 2 and 4 Multi-Mode Microplate Readers

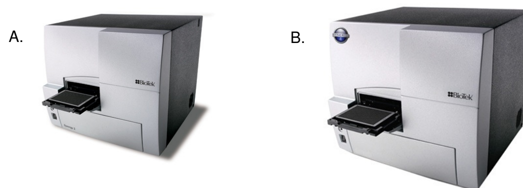


Figure 2. The Synergy 2 and Synergy 4 Microplate Readers. The Synergy 2 and 4 plate readers can be used in well formats from 6- to 1536-well plates. Both readers have 2 broad spectrum light sources for fluorescence. Gen5 Data Analysis Software comes standard with both readers. The Synergy 2 (A) can be customized with combinations of monochromator, filters and dichroic mirrors for optimal performance in all detection modes. The Synergy 4 (B) contains Hybrid Technology comprising a high-performance filter system with flexible monochromator detection. For optimal FP performance, an excitation filter at 620/40 nm was used, combined with an emission filter at 680/30 nm and 660 nm cutoff dichroic mirror. The instrument comes with a standard PMT, or optional red-shifted PMT detector which we tested in this study.

Assay Validation at 10% ATP Conversion

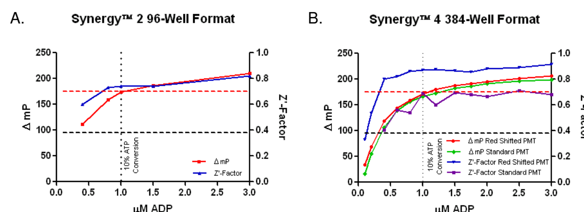


Figure 3. The Synergy 2 and 4 meet BellBrook Labs' Instrument Validation Criteria. The validation criteria include: Z´ ≥ 0.7 at 10% conversion of 10 µM ATP, a Δ mP shift ≥ 95 mP at 10% conversion and read time ≤ 5 minutes. The 96-well format assay passed validation under the following conditions: 15 flashes per well, Z´= 0.74, Δ mP of 174 in 1 minute, 39 seconds. The 384-well format assay passed validation with the red-shifted PMT under the following conditions: 3 flashes per well, Z´= 0.81, Δ mP of 168 in 1 minute, 52 seconds. The 384-well format assay using the standard PMT did not meet the validation criteria of the Program, but results yielded acceptable data by many standards: 12 flashes per well, Z´ ≥ 0.6, Δ mP of 166 in 4 minutes, 11 seconds.

PKA Enzyme Titrations

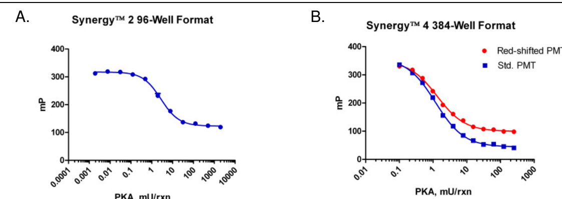


Figure 4. PKA titrations in 96- and 384-well plate formats show comparable EC_{50}. PKA was titrated (1:2) and reactions were performed in a 10 µL assay volume with 10 µM ATP and 50 µM kemptide substrate, and incubated for one hour. Detection Mixture was added and reactions equilibrated for one hour before reading. The final 100 µL (96-well) and 20 µL (384-well) assay volume with the Detection Mixture consisted of 2 nM ADP Alexa633 tracer, 0.5X Stop & Detect Buffer B, 8.5 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, 5 µM ATP and 25 µM kemptide). EC_{50} for 96-well assay was 2.8 mU/rxn. EC_{50} for 384-well assay was 1.3 mU/rxn (red-shifted PMT), and 1.2 mU/rxn (standard PMT).

Z´-Factor at Low % ATP Conversion

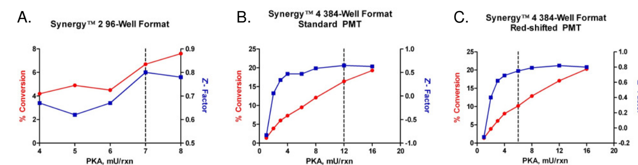
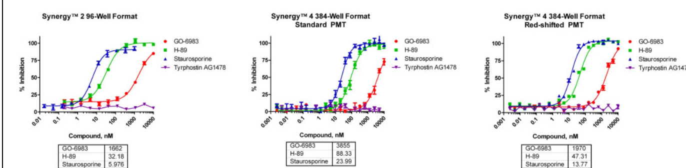


Figure 5. Excellent Z´ values achieved at low % ATP conversion. Z´-factor experiments were run to confirm % conversion achieved in the PKA titration. Enzyme concentrations were chosen as indicated on the graphs above, and reactions were performed under the same conditions used for the enzyme titration. Z´-factor was determined by comparing wells with enzyme to wells without enzyme. Enzyme concentrations chosen for subsequent inhibitor titration experiments achieved a Z´ ≥ 0.7, highlighted by the dashed lines above. 96-well format (A): 7 mU/rxn achieved a Z´= 0.8 at 7% conversion. 384-well format, standard PMT (B): 12 mU/rxn achieved Z´= 0.7 at 16% conversion. 384-well format, red-shifted PMT (C): 6 mU/rxn achieved Z´= 0.8 at 10% conversion.

PKA Inhibitor Potency Study



Synergy™ 2 96-Well Format	
GO-6983	1662
H-89	32.18
Staurosporine	5.976

Synergy™ 4 384-Well Format, Standard PMT	
GO-6983	3855
H-89	88.33
Staurosporine	23.99

Synergy™ 4 384-Well Format, Red-shifted PMT	
GO-6983	1970
H-89	47.31
Staurosporine	13.77

Figure 6. Potency of PKA inhibitors are comparable between well formats. GO-6983, H-89, and staurosporine (known PKA inhibitors) and Tyrphostin AG1478 (a no inhibition control for PKA) were serially titrated into the PKA reaction following the assay conditions noted previously. Results show that the potency of compound inhibition (nM) of the known inhibitors are all comparable between the different well formats tested. There is also good agreement between the inhibitor results obtained in 384-well format using the standard and red-shifted PMT.

Conclusions

- For optimal performance of Transcreener ADP² Assay on BioTek microplate readers, we recommend the following: 620/40 nm excitation filter, 680/30 nm emission filter, 660 nm cutoff dichroic mirror.
- The Synergy 2 and 4 pass BellBrook Labs' Validation Program in 96-well format under the following conditions: standard PMT,15 flashes/well to yield Z´> 0.7 in 1 minute, 39 seconds.
- The Synergy 2 and 4 pass BellBrook Labs' Validation Program in 384-well format under the following conditions: red-shifted PMT, 3 flashes/well to yield Z´> 0.7 in 1 minute, 52 seconds.
- Studies with a PKA model system in 96- and 384-well plate formats show comparable results on the Synergy 2 and 4 microplate readers.

For more information on Transcreener validation on BioTek readers, or for a copy of this poster, please visit BioTek at booth # 443.

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