Application Note

# Optimizing Performance of the Transcreener® ADP Assay for the BioTek Synergy™ 2 and 4 Multi-Mode Microplate Readers

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## In this guide:

- Recommended settings for the BioTek Synergy™ 2 and 4 Multi-Mode Microplate Readers.
- Optimizing sensitivity setting and flash number improves data.
- Synergy™ 2 and 4 meet requirements for BellBrook Labs' Instrument Validation Program when used with the Red-shifted PMT detector.

## Introduction

Transcreener is a universal, high throughput biochemical assay based on detection of nucleotides, which are formed by thousands of cellular enzymes - many of which catalyze the covalent regulatory reactions that are central to cell signaling and are high value targets in drug discovery. The advantages of the Transcreener® HTS Assay Platform over existing assay methods include the following.

***Universality***-The detection of invariant nucleotide reaction product means that a single set of detection reagents can be used for all of the enzymes in a family and all acceptor substrates.

***Far Red Fluorescence Polarization Detection***-Use of far red shifted dyes with a ratiometric output greatly reduces interference and particulate-based light scattering from fluorescent compounds.

***Sensitivity***-High affinity antibodies enable robust detection of low levels of substrate conversion (<10%) with less enzyme than other methods.

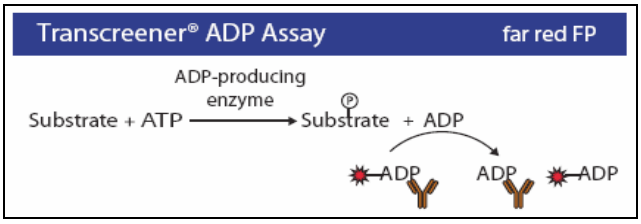
A critical factor in realizing the numerous advantages of the Transcreener HTS assays is the correct setup of the microplate reader.

Proper selection of filters, dichroics, monochromator settings, and read times impact an instrument's sensitivity with any given assay. In response to this fact, BellBrook Labs has developed an Instrument Validation Program to test and optimize plate readers. This will ensure that researchers are aware of the readers meeting minimal performance requirements, as well as knowing the most optimal settings for each detection system.

## Assay Principle

BellBrook Labs' Instrument Validation Program employs the Transcreener® ADP Assay for all testing. The results are representative of those obtained with other Transcreener Far Red FP Assays. The excitation peak is centered at 633nm, while the emission peak centers at 650nm.

The Transcreener ADP assay is a simple one-step homogenous detection assay. Transcreener ADP Detection Mixture, comprised of an ADP Alexa633 Tracer bound to an ADP Antibody, is added to an equal volume of enzyme reaction mix. Enzymatically generated ADP displaces the tracer resulting in a decrease in fluorescence polarization. Standard curves are generated using varying concentrations of ATP and ADP to mimic the conversion of ATP to ADP during the course of an enzyme reaction.





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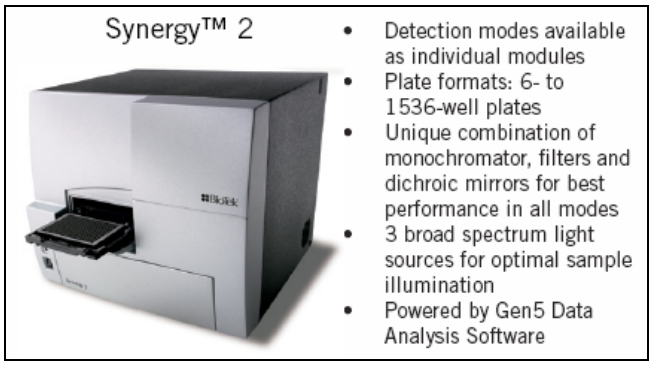
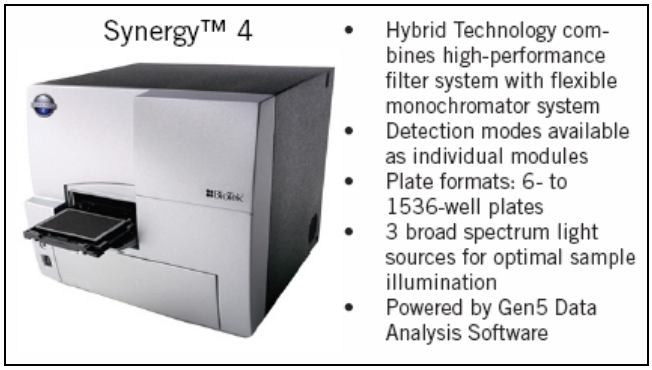
## Validation Criteria

- 384-Well Format
- Z'-Factor ≥ 0.7 at 10% conversion of 10 µM ATP
- Δ mP ≥ 95 mP at 10% conversion of 10 µM ATP
- Z' and Δ mP specifications to be met using Transcreener ADP Assay reagents
- Read time to achieve Z' and Δ mP specifications ≤ 5 minutes

## Synergy™ 2 and Synergy™ 4 Information




## Materials and Methods

Instrument: Synergy™ 4 Hybrid Microplate Reader

Microplates: Corning® 384-Well Low Volume Black Round Bottom PS NBS™ Microplate (Product #3676)

Reagents

| Reagent | Kit/Component Catalog # |
|---|---|
| Transcreener® ADP Assay | 3004-1K |
| ADP Alexa633 Tracer, 400 nM | 2013 |
| Stop & Detect Buffer, 10X | 2015 |
| 500 µM ADP | 2016 |
| ADP Antibody | 2018 |
| 500 µM ATP | Not Provided |
| Buffer Components | |
| 500 mM EGTA | 2039 |
| 1000 mM HEPES | Not Provided |
| 500 mM $MgCl_2$ | |
| 1% Brij-35 | |
| 100% DMSO | Not Provided |

**Table 1. Experimental Reagent**

Protocol

Standard protocol consists of adding 10 µL of ADP Detection Mixture to 10 µL of the ATP/ADP Standard Mixture in a 384-well plate. The plate was then covered, shaken to mix the reagents, and incubated at room temperature for 60 minutes.

**ATP/ADP Mixture**
The ATP/ADP mixture consists of 4 mM MgCl2, 2 mM EGTA, 50 mM HEPES, pH 7.5, 1% DMSO, 0.01% Brij-35, and ATP/ADP combined to a constant adenine concentration of 10 µM.

**ADP Detection Mixture**
The ADP Detection Mixture consists of 1X Stop & Detect Buffer, 4 nM ADP Far Red Tracer, and 20 µg/mL ADP Antibody.

**Free Tracer**
The Free Tracer consists of 1X Stop & Detect Buffer, and 4 nM ADP Far Red Tracer.

**Buffer Blank**
The Buffer Blank consists of 1X Stop & Detect Buffer, and 20 µg/mL ADP Antibody.

**Final Concentrations in 20 µL Reaction Volume**
2 mM MgCl2, 1 mM EGTA, 25 mM HEPES (pH 7.5), 0.5% DMSO, 0.005% Brij-35, ATP/ADP combined to a constant adenine concentration of 5 µM, 0.5X Stop & Detect Buffer (25 mM HEPES, pH 7.5, 200 mM NaCl, 10 mM EDTA, and 0.01% Brij-35), 2 nM ADP Far Red Tracer, and 10 µg/mL ADP Antibody.

**Standard Curve Preparation**

15-point ATP/ADP standard curves were generated to test the Synergy™ 4 Hybrid Microplate Reader. ATP/ADP mixtures were created at the various concentrations of ATP and ADP listed in Table 2. Final concentration of the buffer components are listed above. Each point on the curve mimics a different substrate conversion level in an enzyme reaction (n=24). 10 µL of each ATP/ADP combination was dispensed across an entire row of a 384-well plate. 10 µL of the 10 µM ATP/0 µM ADP combination was also dispensed to row P of the plate.

10 µL of the prepared ADP Detection Mixture was then dispensed to rows A-O of the assay plate. Finally, in place of the ADP Detection Mixture, 10 µL of free tracer was dispensed to wells P1-P12, and 10 µL of buffer blank was dispensed to wells P13-P24.

| Substrate Conversion Levels (%) | ATP, µM | ADP, µM |
|---|---|---|
| 0 | 10 | 0 |
| 1 | 9.9 | 0.1 |
| 2 | 9.8 | 0.2 |
| 4 | 9.6 | 0.4 |
| 6 | 9.4 | 0.6 |
| 8 | 9.2 | 0.8 |
| 10 | 9 | 1 |
| 12 | 8.8 | 1.2 |
| 15 | 8.5 | 1.5 |
| 17.5 | 8.25 | 1.75 |
| 20 | 8 | 2 |
| 25 | 7.5 | 2.5 |
| 30 | 7 | 3 |
| 60 | 4 | 6 |
| 100 | 0 | 10 |

**Table 2. Standard Curve ATP/ADP Concentrations**

Instrument Set-up and Filter Information

| Instrument Detection Components | | BioTek Catalog # |
|---|---|---|
| Excitation Filter | 620/40nm | 7082213 |
| Emission Filter | 680/30nm | 7082229 |
| Dichroic | 660nm cutoff | 7137660 |
| PMT | Standard PMT detector | 49984 |
| PMT | Red-shifted PMT detector | 49721 |

**Table 3. Instrument Optics**

The excitation and emission filters, and dichroic were installed prior to instrument evaluation. The standard PMT was previously installed in the instrument, while the Red-shifted PMT detector was installed during the evaluation process. Following the installation process, and prior to the first read of a plate, the following steps were taken to optimize the detector gain with the use of the Sensitivity setting, which prepared the Synergy 4 to correctly read the Transcreener® ADP Assay:

1. The Read Step was opened within the Procedure to be used to read the plate.
2. The Sensitivity “Options” was chosen, and “Automatic Sensitivity Adjustment” and “Scale to High Wells” were selected.
3. A well containing free tracer was chosen, and the “Scale Value” was set to 50,000.

This function allows the user to select a well or a group of wells on the plate, and have the reader scale the raw measurement to these wells. The recommended “Scale Value” is 50,000 RFU. This value allows for the use of the full measurement range of the system, while leaving some room for samples that might be a little brighter than expected. Typically, the wells used for the scaling are positive controls, the highest standards of the standard curve, or any other well expected to provide a strong signal. Subsequent reads using the same plate used the predetermined sensitivity settings. To ascertain this value the following steps were completed:

1. The Procedure options were opened by right clicking on “Procedure”.
2. “AutoSensitivity Results” was chosen. The sensitivity setting was recorded from the “AutoSensitivity Results” screen.
3. The Read Step was once again opened, and the Sensitivity Options was chosen.
4. The “Automatic Sensitivity Adjustment” option was deselected. This allowed the value used for the initial read to be entered into the “Sensitivity” box.

| Optimized Measurement Settings | |
|---|---|
| Sensitivity Setting | 168 |
| Top Probe Vertical Offset | 7.00 |
| Flash Number | variable |

**Table 4. Instrument Settings**

Fluorescence polarization measurements were performed using the settings listed in Table 4. The sensitivity setting was optimized prior to reading the plate, using the procedure previously described. The number of flashes per well and top probe vertical offset were manually adjusted in the Read Step of the Protocol. Flash number was varied to determine the range of read times that would meet the criteria of the instrument validation program.

## Calculations

### Δ mP Calculation

mP values for each substrate percent conversion level were subtracted from the mP value at 0% ATP conversion.

$mP = mP_{initial\ [ATP]} - mP_{sample}$

The change in mP values (Δ mP) is indicative of the amount of ATP that is converted to ADP in an enzyme reaction. A Δ mP of approximately 100 mP units is ideal in a compound screening situation.

### Z'-Factor Calculation

$Z' = 1 - [(3*SD_{initial\ [ATP]} + 3*SD_{sample})/|(mP_{initial\ [ATP]} - mP_{sample})|]$

While an assay yielding a Z' ≥ 0.5 is considered a high quality assay, those producing Z' values ≥ 0.7 give the user a greater confidence level.

## Results

Assay plates containing the 15-point standard curve, generated using 2 nM ADP Alexa633 Tracer, were read on the Synergy™ 4 Hybrid Microplate Reader (Figure 1). As the ratio of ADP: ATP increases the proportion of bound tracer vs. free tracer decreases resulting in an overall decrease in mP values.

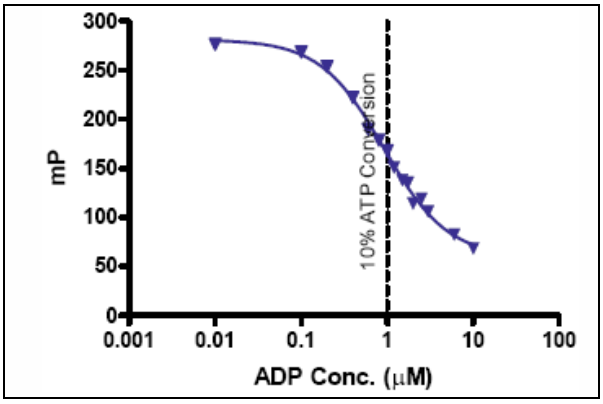
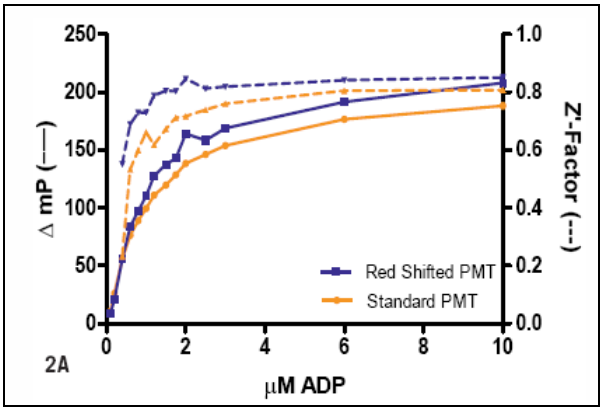



**Figure 1. 10 μM ATP/ADP Standard Curve. 10% ATP Conversion represents 9 μM ATP/1 μM ADP concentration level**.



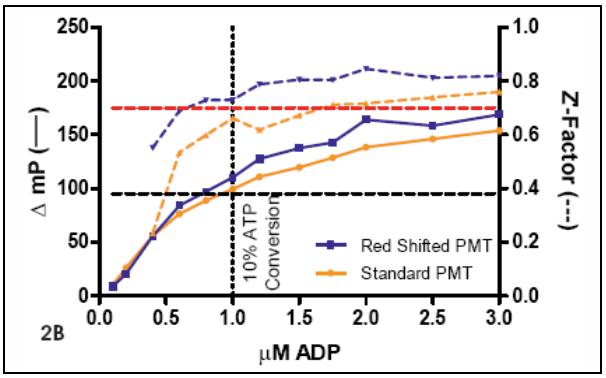




**Figure 2. A) Z' and Δ mP values observed in a standard curve mimicking conversion of 10 μM ATP to ADP. B) A zoom in of the 1-3 μM ADP section of the standard curve. Z' validation minimal qualification shown by red dashed line. Δ mP validation minimal qualification shown by black dashed line. 10% ATP conversion validation point shown by black dotted line. Reader set at 15 flashes when using Standard PMT, and 8 flashes when using Red-Shifted PMT**

A Z'>0.7 and an mP shift >100 units is achieved at 1.0 µM ADP (10% ATP conversion) in a read time of 3:10 using the Red-Shifted PMT (Figure 2A). The Z' value falls below 0.7 at 6% substrate conversion, which coincides with a Δ mP value of 84. Figure 2B highlights data that is generated in the initial velocity range of the reaction. Validation criteria are met by the Synergy™ 4 using a reader setting of 8 flashes using the Red-Shifted PMT.

| Assay Performance at 10% Conversion of 10 µM ATP | | | | | |
|---|---|---|---|---|---|
| **Results with Standard PMT** | | | | | |
| **Flashes** | | | **10** | **12** | **15** |
| **Read Time (Minutes)** | | | **3:41** | **4:11** | **4:57** |
| 10% ATP Conversion ΔmP | | | 98 | 98 | 100 |
| 10% ATP Conversion Std. Dev. | | | 6 | 8 | 5 |
| 10% ATP Conversion Z'-Factor | | | 0.58 | 0.61 | 0.66 |
| **Results with Red-Shifted PMT** | | | | | |
| **Flashes** | **5** | **8** | **10** | **12** | **15** |
| **Read Time (Minutes)** | **2:22** | **3:10** | **3:41** | **4:11** | **4:57** |
| 10% ATP Conversion ΔmP | 111 | 110 | 108 | 110 | 109 |
| 10% ATP Conversion Std. Dev. | 7 | 5 | 5 | 4 | 4 |
| 10% ATP Conversion Z'-Factor | 0.63 | 0.73 | 0.75 | 0.77 | 0.79 |

**Table 5. Assay performance with various instrument settings.**

## Conclusions

Variable flash numbers were evaluated to determine the optimal read time generating the highest quality data. As flash number increases, the standard deviation of the mean FP values decreases resulting in improved Z' values (Table 5). The shortest plate read that results in data meeting validation criteria occurs with 8 flashes using the Red-Shifted PMT.

## Discussion

The data shows that the BioTek Synergy™ 2 and 4 are compatible with the Transcreener Far Red FP Detection Module. Even though data was only generated on the Synergy™ 4, due to the fact that the instruments use the same filter based reading platform allows for this conclusion. Following the Transcreener protocol with the use of the standard PMT, and setting the instrument to 12 flash reads or above will yield Z' values ≥ 0.6. While this is acceptable data by many standards, the Z' values can be improved and the instrument meets validation criteria with the use of the Red-Shifted PMT. Using this format, setting the instrument to 8 flash reads will yield Z' values ≥ 0.7 in a read time of 3:10. It is important to use the instrument setup described in the materials and methods. A change in filters or dichroics may have adverse effects on instrument performance resulting in an increased standard error in reads.

- BioTek Synergy™ 4 Hybrid Microplate Reader passed the validation criteria under the following conditions: Using a 620/40 nm excitation filter, a 680/30 nm emission filter, a 660nm cutoff dichroic, and a Red-Shifted PMT; instrument was set to 8 flashes and yielded a Z' >0.7 in 3:10 min.
- BioTek Synergy™ 2 also passes validation criteria due to similar filter based detection platform.
- Using optimized instrument settings and the filters and dichroic recommended by BioTek reduces standard error in mP reads.



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