

In this guide:

- Recommended settings for the PerkinElmer EnVision® using the ADP<sup>2</sup> Assay
- Optimizing gain settings and flash number improves data
- EnVision® meets requirements for BellBrook Labs' Instrument Validation Program



## Introduction

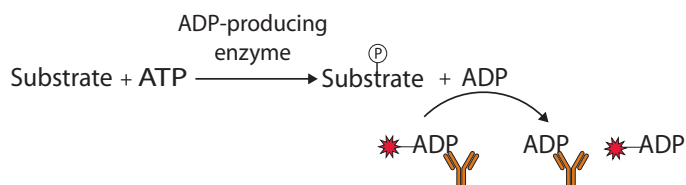
Transcreener is a universal, high throughput biochemical assay based on detection of nucleotides, which are formed by thousands of cellular enzymes - many of which catalyze the covalent regulatory reactions that are central to cell signaling and are high value targets in drug discovery. The advantages 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<sup>2</sup> Assay for all testing. The excitation peak is centered at 633nm, while the emission peak centers at 650nm. The Transcreener ADP<sup>2</sup> assay is a simple one-step homogenous detection assay. Transcreener ADP Detection Mixture, comprised of an ADP Alexa633 Tracer bound to an ADP<sup>2</sup> 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.

### Transcreener® ADP<sup>2</sup> FP Assay far red FP



## Validation Criteria

- 384-Well Format
- Z'-Factor  $\geq 0.7$  at 10% conversion of 10  $\mu\text{M}$  ATP
- $\Delta$  mP  $\geq 95$  mP at 10% conversion of 10  $\mu\text{M}$  ATP
- Z' and  $\Delta$  mP specifications to be met using Transcreener® ADP<sup>2</sup> Assay reagents
- Read time to achieve Z' and  $\Delta$  mP specifications  $\leq 5$  minutes

The results from the validation using the Transcreener® ADP<sup>2</sup> Assay are representative of the performance with other Transcreener Far Red FP Assays with this instrument. Because individual results may vary, optimal setting recommendations should be used as a starting point. Slight modifications may be necessary to optimize results for your particular assay on your instrument.

## EnVision® Information



- Reads Fluorescence Polarization and TR-FRET versions of Transcreener assays
- Capable of reading 96, 384, and 1536-well assay plates
- Simultaneous dual-detection capabilities
- Interchangeable filters and dichroic modules

## Materials and Methods

**Instrument:** EnVision® 2100 Multilabel Plate Reader

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

### Reagents

| Reagent                                 | Kit/Component Catalog # |
|---|-------------------------|
| Transcreener® ADP <sup>2</sup> FP Assay | 3010-1K                 |
| ADP Alexa633 Tracer, 400 nM             | 2009                    |
| Stop & Detect Buffer B, 10X             | 2027                    |
| ADP <sup>2</sup> Antibody               | 2051                    |
| 5 mM ADP                                | 2052                    |
| 5 mM ATP                                | 2053                    |
| Buffer Components                       |                         |
| 500 mM EGTA                             | Not Provided            |
| 1000 mM HEPES                           |                         |
| 500 mM MgCl <sub>2</sub>                |                         |
| 1% Brij-35                              |                         |
| 100% DMSO                               |                         |

**Table 1. Experimental Reagents**

### 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 MgCl<sub>2</sub>, 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 B, 4 nM ADP Alexa633 Tracer, and 14.8 µg/mL ADP<sup>2</sup> Antibody.

### Free Tracer

The Free Tracer consists of 1X Stop & Detect Buffer B, and 4 nM ADP Alexa633 Tracer.

### Buffer Blank

The Buffer Blank consists of 1X Stop & Detect Buffer B, and 14.8 µg/mL ADP<sup>2</sup> Antibody.

### Final Concentrations in 20 µL Reaction Volume

2 mM MgCl<sub>2</sub>, 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 B (25 mM HEPES, pH 7.5, 200 mM NaCl, 10 mM EDTA, and 0.01% Brij-35), 2 nM ADP Alexa633 Tracer, and 7.4 µg/mL ADP<sup>2</sup> Antibody.

### Standard Curve Preparation

15-point ATP/ADP standard curves were generated to test the EnVision® Multilabel Plate 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

| Ex. Filter/Em. Filter/Dichroic |                                      | Perkin Elmer Catalog # |
|--------------------------------|--------------------------------------|------------------------|
| Package                        | Optimized Cy5 FP Dual Emission Label | 2100-8390              |
| Excitation Filter              | 620/40 nm                            | 2100-5760              |
| Emission Filters               | 688/45 nm                            | 2100-5780/5790         |
| Mirror                         | D658/fp688 dual mirror               | 2100-4260              |

**Table 3. Instrument Optics**

The following steps were taken to prepare the EnVision® to correctly read the Transcreener® ADP<sup>2</sup> FP Assay:

1. A label was created by replicating an existing label, after the installation of the filters and dichroic listed in Table 3.
2. The correct filters and mirror that were installed in the instrument were associated with the new label, in the "General" tab.
3. A new protocol was created using the same replication process mentioned above, and the label and plate to be used were associated with the protocol.
4. The label to be used to read the plate was optimized using the Label Optimization Wizard. The correct protocol was chosen, and then "Plate Dimension", "Measurement Height", and "Detector Gains" were chosen to be optimized.
5. Following completion of the wizard, the G-Factor and measurement height were recorded from the "Optimization" tab for the label. The optimization was then deleted. The G-Factor and measurement height information was then recorded in the "General" tab for that label.
6. The Label Optimization Wizard was run a second time, with only "Plate Dimension" being chosen for optimization. This once again sends the EnVision® the correct plate dimensions for the test plate, while allowing the detector gains to be increased above the recommended settings of the reader.

| Optimized Measurement Settings |          |
|--------------------------------|----------|
| Detector Gain 1                | 800      |
| Detector Gain 2                | 800      |
| Measurement Height             | 12.0     |
| G-Factor                       | 0.62     |
| Excitation Light (%)           | 100      |
| Flash Number                   | variable |

**Table 4. Instrument Settings**

Fluorescence polarization measurements were performed using the settings listed in Table 4. The G-Factor, Measurement Height, and Excitation Light % were optimized prior to reading the plate, using the procedure previously described. The number of flashes per well and Detector Gains were manually adjusted in the appropriate label. 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.

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

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.

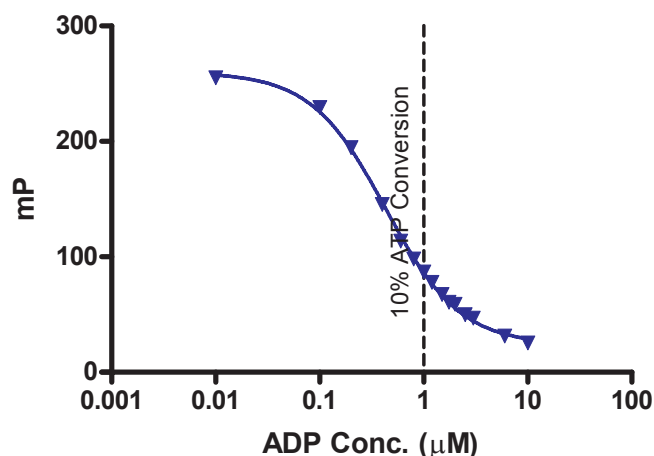
#### Z'-Factor Calculation

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

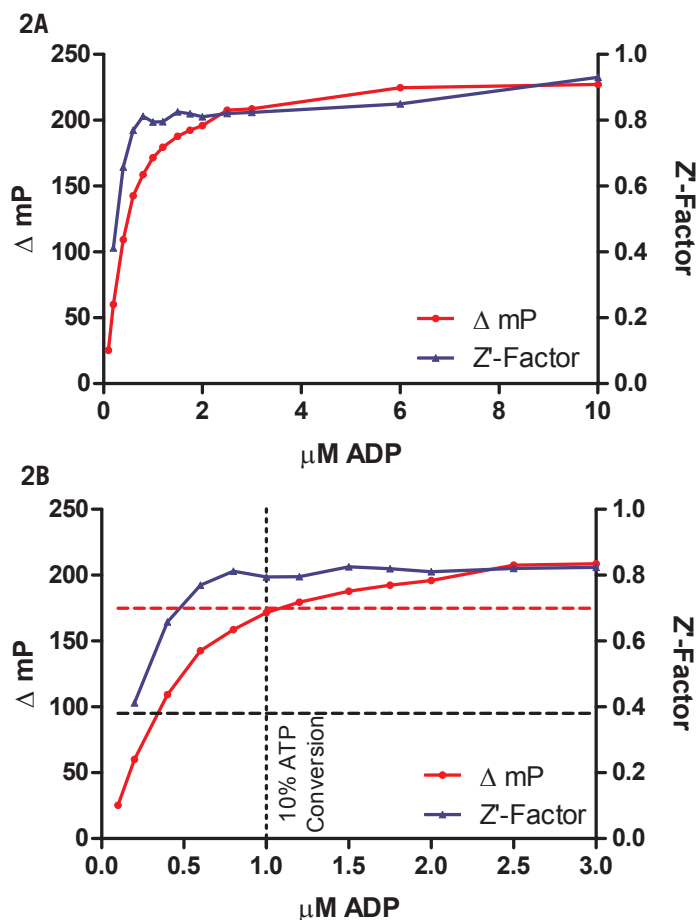
While an assay yielding a Z' ≥ 0.5 is considered a high quality assay, those producing Z' values ≥ 0.7 give the user a greater confidence level.

## Results

Assay plates containing the 15-point standard curve were read on the EnVision® Multilabel Plate 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.**



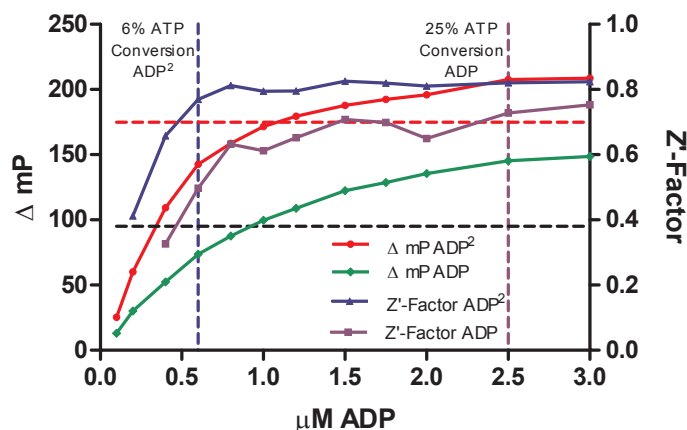
**Figure 2. A) Z' and Δ mP values observed in a standard curve mimicking conversion of 10 μM ATP to ADP. B) A zoom in of the 1-3 μM ADP section of the standard curve. Z' validation minimal qualification shown by red dashed line. Δ mP validation minimal qualification shown by black dashed line. 10% ATP conversion validation point shown by black dotted line. Reader set at 30 flashes.**

A Z' > 0.7 and an mP shift of 172 units is achieved at 1.0 μM ADP (10% ATP conversion) in a read time of 1:20 (Figure 2A). The Z' value falls below 0.7 at 4% substrate conversion, which coincides with a Δ mP value of 109. Figure 2B highlights data that is generated in the initial velocity range of the reaction. Validation criteria are met by the PerkinElmer EnVision® using a reader setting of 30 flashes.

| Assay Performance at 10% Conversion of 10 μM ATP |      |      |      |      |      |      |
|--|------|------|------|------|------|------|
| Flashes  | 30   | 50   | 75   | 100  | 150  | 200  |
| Read Time (Minutes)                              | 1:20 | 1:37 | 2:00 | 2:17 | 3:01 | 3:43 |
| 10% ATP Conversion ΔmP                           | 172  | 173  | 170  | 170  | 168  | 167  |
| 10% ATP Conversion Std. Dev.                     | 6    | 5    | 4    | 3    | 4    | 3    |
| 10% ATP Conversion Z'-Factor                     | 0.79 | 0.83 | 0.86 | 0.88 | 0.87 | 0.89 |

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

Variable flashes 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 slightly, resulting in improved Z' values (Table 5). The shortest plate read that results in data meeting validation criteria occurs with 30 flashes.



**Figure 3. Z' and  $\Delta$  mP value comparison between the Transcreener<sup>®</sup> ADP and ADP<sup>2</sup> FP assays. Z' validation minimal qualification shown by red dashed line.  $\Delta$  mP validation minimal qualification shown by black dashed line. Lowest % ATP conversion level yielding Z' > 0.7 for ADP assay shown by purple dashed line. Lowest % ATP conversion level yielding Z' > 0.7 for ADP<sup>2</sup> assay shown by blue dashed line. Reader set at 200 flashes for the ADP assay, and 30 flashes for the ADP<sup>2</sup> Assay.**

Data quality between the Transcreener<sup>®</sup> ADP FP Assay and ADP<sup>2</sup> FP Assay can be compared by examining the Z' and  $\Delta$  mP values generated at various % ATP conversion levels within a standard curve. Results show that the Transcreener<sup>®</sup> ADP<sup>2</sup> FP Assay is able to yield higher quality data at conversion levels that lie well within initial rate enzyme reaction kinetics. The Transcreener<sup>®</sup> ADP<sup>2</sup> FP Assay enables much shorter read times than the original assay on this instrument.

## Discussion

The data shows that the PerkinElmer EnVision<sup>®</sup> is compatible with the Transcreener Far Red FP Detection Module. Following the Transcreener protocol and setting the instrument to 30 flashes yielded Z' values  $\geq 0.7$  in a read time of 1:20. The Z' can be improved by increasing the number of flashes at the expense of shorter read times. The data also shows that the Transcreener<sup>®</sup> ADP<sup>2</sup> FP Assay improves data quality at low % ATP conversion levels, thereby making it possible to use smaller amounts of enzyme and substrate in an enzyme reaction. It is important to use the instrument setup described in the materials and methods. A change in settings may have adverse effects on instrument performance resulting in an increased standard error in reads.

## Conclusions

- PerkinElmer's EnVision<sup>®</sup> Multilabel Plate Reader passed the validation criteria under the following conditions: Optimized Cy5 FP Dual Emission Label combined with 30 instrument flashes. These settings yielded a Z' > 0.7 with a read time of 1:20 minutes
- Using optimized instrument setup recommended by PerkinElmer reduces standard error in mP reads
- Transcreener<sup>®</sup> ADP<sup>2</sup> FP Assay improves data quality using initial rate enzyme reaction kinetics

## Additional Information

### Related Products

|   |         |
|---|---------|
| Transcreener <sup>®</sup> ADP <sup>2</sup> FP Assay.....          | 3010-1K |
| Transcreener <sup>®</sup> ADP <sup>2</sup> TR-FRET Red Assay..... | 3011-1K |
| Transcreener <sup>®</sup> ADP <sup>2</sup> FI Assay.....          | 3013-1K |
| Transcreener <sup>®</sup> AMP/GMP Assay.....                      | 3006-1K |
| Transcreener <sup>®</sup> UDP Assay.....                          | 3007-1K |
| Transcreener <sup>®</sup> GDP FP Assay.....                       | 3009-1K |

### Ordering Information

Please contact BellBrook Labs for product pricing. Custom quotes are available for orders of 10,000 wells or more.

Phone orders:  
608.443.2400  
866.313.7881

Fax orders:  
608.441.2967

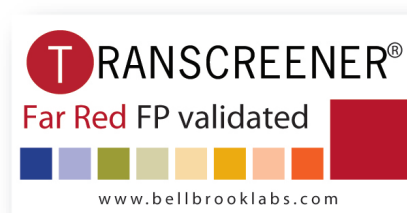
Email orders:  
info@bellbrooklabs.com

Transcreener<sup>®</sup> HTS Assay Platform is a patented technology of BellBrook Labs. Transcreener<sup>®</sup> is a registered trademark of BellBrook Labs. AlexaFluor<sup>®</sup> is a registered trademark of Molecular Probes, Inc (Invitrogen).

### Technical Information

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### Transcreener Instrument Validation Stickers



Look for the Transcreener Far Red FP-validated sticker on instruments that have successfully met our validation criteria.