

# Application Note

## In this guide:

### Setting recommendations using the Tecan Infinite® F200 for the FI Assays

#### 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 Intensity Detection

Use of far red shifted dyes with a simple relative fluorescence output greatly reduces interference and particulate-based light scattering from fluorescent compounds.

##### Sensitivity

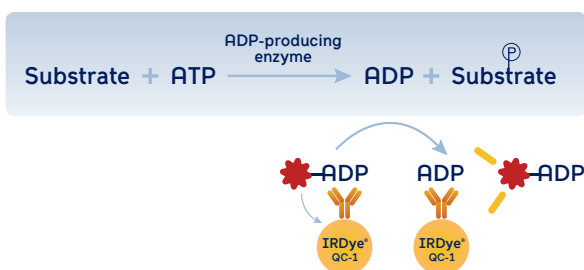
High affinity antibodies enable robust detection of low levels of substrate conversion (<10%) with less enzyme than other methods.

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. **Note that the settings are the same for ADP<sup>2</sup> and GDP FI assay but we have specifically demonstrated the performance using ADP/ATP as the experimental nucleotide.** In this application note we determine the impact of key instrument parameters on Transcreener assay performance. Using this information the researcher can select the instrument set-up most appropriate for their application.

#### Assay Principle

The Transcreener ADP<sup>2</sup> FI Assay is a simple one-step homogenous detection assay. The Transcreener ADP Detection Mixture comprises an ADP Alexa594 Tracer bound to the ADP<sup>2</sup> Antibody-IRDye® QC-1 quencher conjugate (quencher licensed from LI-COR®). ADP<sup>2</sup> produced by the target enzyme displaces the tracer from the Ab-quencher conjugate which causes an increase in fluorescence. The use of a red tracer minimizes interference from fluorescent compounds and light scattering.

#### Transcreener® ADP<sup>2</sup> FI Assay Principle



#### Performance Criteria

- 384-Well Format
- Z'-Factor ≥ 0.7 at 10% conversion of 10 μM ATP

Standard curves were generated using varying concentrations of ATP and ADP to mimic the conversion of ATP to ADP during the course of an enzyme reaction. Instrument settings were optimized to read the results of the standard curves. 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.

#### Infinite® M200 Information

- **Quad-4 Monochromators™ technology .**
- **Eight Detection modes.**
- **Fast measurements.**
- **Equipped with Injector module.**
- **High measurement speed and robotic compatibility -** Infinite® M-200 is able to handle microplate format up to 384-wells with high speed in every detection mode.



## Materials

**Instrument:** Infinite<sup>®</sup> M-200 Microplate Reader

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

Reagent	Kit/Component Catalog #
Transcreener <sup>®</sup> ADP <sup>2</sup> FI Assay	3013-1K
ADP Alexa594 Tracer, 800 nM	2073
Stop & Detect Buffer B, 10X	2027
ADP <sup>2</sup> Antibody-IRDye <sup>®</sup> -QC-1	2074
5mM 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, 8 nM ADP Alexa594 Tracer, and 10 µg/mL of ADP<sup>2</sup> Antibody-IRDye<sup>®</sup> QC-1.

#### Free Tracer

The Free Tracer consists of 1X Stop & Detect Buffer B, and 8 nM ADP Alexa594 Tracer.

#### Buffer Blank

The Buffer Blank consists of 1X Stop & Detect Buffer B, and 10 µg/mL ADP<sup>2</sup> Antibody-IRDye<sup>®</sup> QC-1.

#### 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), 4 nM ADP Alexa594 Tracer, and 5 µg/mL of ADP<sup>2</sup> Antibody-IRDye<sup>®</sup> QC-1.

#### Standard Curve Preparation

15-point ATP/ADP standard curves were generated to test the Infinite<sup>®</sup> M-200 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 buffer 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
0.5	9.95	0.05
1	9.90	0.1
2	9.8	0.2
4	9.60	0.4
6	9.40	0.6
8	9.2	0.8
10	9.0	1
15	8.5	1.5
20	8.0	2.0
30	7	3
40	6	4
60	4	6
100	0	10

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

### Instrument Set-up and Filter Information

Optimized Instrument Wavelengths	
Excitation Wavelength and Bandwidth	585/9nm
Emission Wavelength and Bandwidth	627/20nm

**Table 3. Instrument Optics**

When using the I-Control<sup>™</sup> software, the Infinite<sup>®</sup> M-200 can be set to run the ADP<sup>2</sup> FI Assay by performing the following steps:

1. Choose "Edit Measurement Parameters" from the i-control<sup>™</sup> dropdown menu.
2. In the General tab, select "Fluorescence Intensity", and Endpoint.
3. In the Plate tab, select the correct Plate definition file for the assay plate that will be used when running the assay. If only part of the plate will be read, check "Part of the plate" and then select the well range that will be read from the dropdown menus.
4. In the Wavelengths tab, select "Fixed Wavelength", and then select "Other" from the dropdown menu. In the Excitation wavelength dropdown menu, select 585 nm. In the Excitation bandwidth dropdown menu, select 9nm. In the Emission wavelength dropdown menu, select 627 nm. In the Emission bandwidth dropdown menu, select 20.0 nm..
5. In the Meas. Params tab, if the plate is being read for the first time, select "optimal" for the Gain. Set the integration time to 20 µs. The "Number of reads:" will be entered by the user, depending on the data quality desired. Click "OK" when finished.
6. Click "Reference-blank" and then select the range of wells on the plate that contain the buffer blank from the dropdown menus.
7. Click "Sample'blank" for Blank reduction, and select "same as reference blank". Click "OK" when finished.

## Optimized Measurement Settings

Plate Definition	User Defined
Integration time	20 $\mu$ s
Lag time	0 $\mu$ s
Flash Number	variable
Gain	Optimal
Settle Time	0 ms

**Table 4. Instrument Settings**

Fluorescence intensity measurements were performed using the settings listed in Table 4. The plate definition and Integration time were manually selected, while all other settings were determined by the instrument. Flash number was varied to determine the range of read times that would meet the criteria of the instrument validation program.

## Calculations

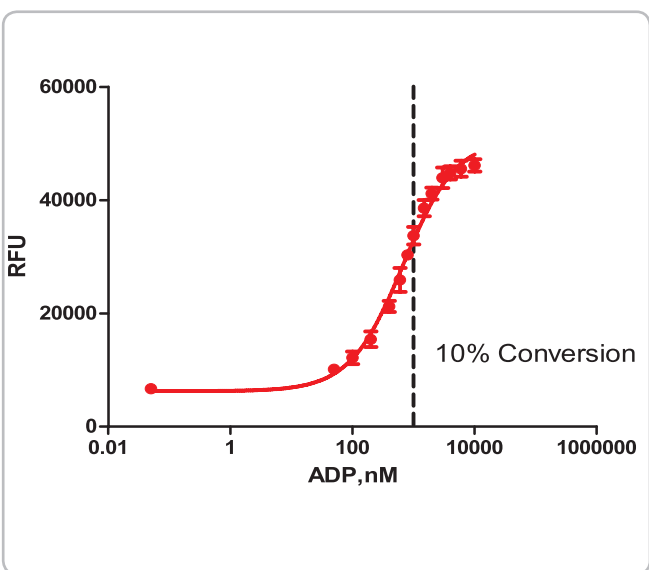
### Z'-Factor Calculation

$$Z' = 1 - \frac{[(3 * SD_{X\% CONV} + 3 * SD_{0\% CONV}) / (RFU_{X\% CONV} - RFU_{0\% CONV})]}{1}$$

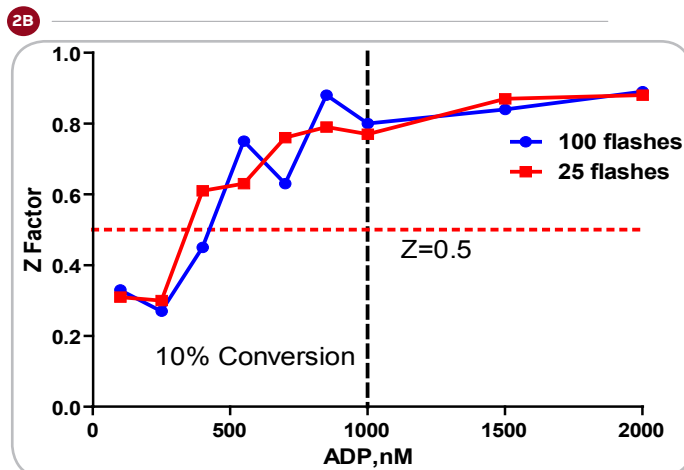
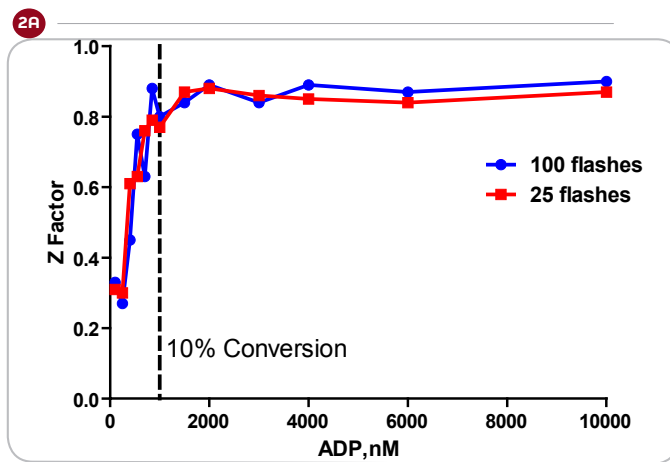
While an assay yielding a  $Z' \geq 0.5$  is considered a high quality assay, those producing  $Z'$  values  $\geq 0.7$  give the user a greater confidence level.

## Results

Assay plates containing the 14-point standard curve were read on the Infinite<sup>®</sup> M-200 Microplate Reader (Figure 1). As the ratio of ADP:ATP increases the proportion of bound tracer vs. free tracer decreases resulting in an overall increase in RFU values.



**Figure 1. 10  $\mu$ M ATP/ADP Standard Curve. 10% ATP Conversion represents 9  $\mu$ M ATP/1  $\mu$ M ADP concentration level.**



**Figure 2. A)  $Z'$  observed in a standard curve mimicking conversion of 10  $\mu$ M ATP to ADP. B) A zoom in of the 0-2  $\mu$ M ADP section of the standard curve.  $Z'$  validation minimal qualification shown by red dashed line. 10% ATP conversion validation point shown by black dotted line. Reader set at 1 flash or 10 flashes.**

A  $Z' > 0.7$  is achieved at 1.0  $\mu$ M ADP (10% ATP conversion) in a read time of 2:00 minutes (Figure 2A). The  $Z'$  value falls below 0.5 at 0.5% substrate conversion. Figure 2B highlights data that is generated in the initial velocity range of the reaction. Validation criteria are met by the Infinite<sup>®</sup> M-200 using a reader setting of 25 flashes.

### Assay Performance at 10% Conversion of 10 $\mu$ M ATP

Flashes	25	100
Read Time (Minutes)	2:00	5:00
% CV at 10% ATP Conversion	4.84	4.62
$Z'$ -Factor at 10% ATP Conversion	0.77	0.80

**Table 5. Assay Performance at Various Instrument Settings**

Variable flashes were evaluated to determine the optimal read time generating the highest quality data. As flash number increases, the percent CV of the mean RFU values decreases slightly, resulting in improved  $Z'$  values (Table 5).

## Discussion

The data shows that the Transcreener ADP<sup>2</sup> FI and GDP FI Assays are sensitive at low percent ATP conversion levels, thereby making it possible to use smaller amounts of enzyme and substrate in an enzyme reaction. Following the Transcreener protocol and setting the instrument to 25 flashes yielded Z' values  $\geq 0.7$  in a read time of 2:00 minutes. The data also shows that the Transcreener ADP<sup>2</sup> FI Assay improves data quality at low percent ATP conversion levels, thereby making it possible to use smaller amounts of enzyme and substrate in an enzyme reaction.

## Conclusions

Tecan Infinite® F-200 met the performance criteria under the following conditions: Using **excitation wavelength of 585 nm with a bandwidth of 9 nm and emission wavelength of 627nm with a 20nm bandwidth and the instrument set to 25 flashes yielded a Z' >0.7 in 2:00 min.**

Using optimized instrument settings with Tecan Infinite® M-200 reduces standard error in RFU measurements.

Transcreener ADP<sup>2</sup> FI assay improves data quality using initial rate enzyme reaction kinetics.

# Additional Information

## Related Products

Transcreener® ADP <sup>2</sup> FP Assay.....	3010-1K
Transcreener® ADP <sup>2</sup> TR-FRET Red Assay.....	3011-1K
Transcreener® ADP <sup>2</sup> FI Assay.....	3013-1K
Transcreener® AMP/GMP Assay.....	3006-1K
Transcreener® UDP Assay.....	3007-1K
Transcreener® GDP FP Assay.....	3009-1K
Transcreener® GDP FI Assay.....	3014-1K

## Ordering

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

## Technical Information

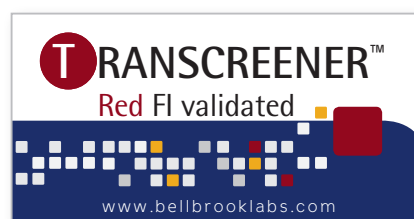
For technical information, please contact  
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## Transcreener Instrument Validation Stickers



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

Transcreener® HTS Assay Platform is a patented technology of BellBrook Labs. Transcreener® is a registered trademark of BellBrook Labs. AlexaFluor® is a registered trademark of Molecular Probes, Inc (Invitrogen). IRDye QC-1® is a registered trademark of LI-COR.