Optimizing Performance of the Transcreener® ADP Assay for the Tecan Infinite® F500 Microplate Reader

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In this guide:

- Recommended settings for the Tecan Infinite® F500 Filter-Based Microplate Reader
- Optimizing gain setting and flash number improves data
- Infinite® F500 meets requirements for BellBrook Labs' Instrument Validation Program

Introduction

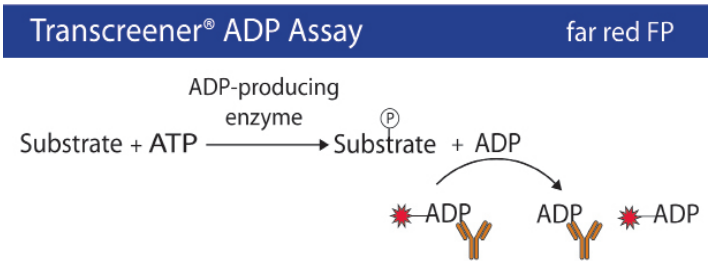
Transcreener is a universal, high throughput biochemical assay based on detection of nucleotides, which are formed by thousands of cellular enzymes - many of which catalyze the covalent regulatory reactions that are central to cell signaling and are high value targets in drug discovery. The advantages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



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

Infinite® F500 Information

- **Injectors -** Optionally equipped with 2 injectors integrated into the system. Syringes can be selected in different volumes, 500 µL, 1000 µL and 2500 µL.
- **Automated z-focus -** Allows for automatic adjustment of z-focus in all top reading modes, significantly improving the quality of measurement data. The automated z-focusing makes it easy to optimize the setup when you need to adapt the instrument settings for highly demanding assay parameters, such as well volume and different well shapes.
- **On-chip storage of filter information -** Programmable filter slides with electronic identity chip.
- **High measurement speed and robotic compatibility -** Capable of reading 6- to 384-well plates, including standard and low volume 384-well plates. All top reading modes can be used for 1536 wells and the Infinite® F500 can be easily integrated into the Freedom EVO® series of automated platforms

Materials and Methods

Instrument: Infinite® F500 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 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 $MgCl_2$, 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 $MgCl_2$, 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 Infinite® F500 Microplate Reader. ATP/ADP mixtures were created at the various concentrations of ATP and ADP listed in Table 2. Final concentrations 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 Information

Instrument Detection Components		Catalog #
Excitation Filter	610/20nm	For detailed information contact the local Tecan sales representative
Emission Filter	670/25nm	
Dichroic Mirror	630nm	

Table 3. Instrument Optics

When using the Tecan i-control™ software, the Infinite® F500 can be setup to run the Transcreener® assay by performing the following steps:

1. Open i-control™, connect to the Infinite® F500 reader and open a new method file.

2. Pick the labware/plate that will be used in the assay from the "Plate definition" dropdown menu.

3. Select the portion of the labware/plate to be read.

4. From the "Measurements" menu, select "Fluorescence Polarization", and insert into the new method.

5. In the "Wavelength" section, select 610 (20) nm from the "Excitation" dropdown menu, and 670 (25) nm from the "Emission:" dropdown menu. Note: The corresponing wavelengths will only be visible if the filters have been defined previously (please refer to the Infinite® F500 operating manual).

6. In the "Mirror" section, select Dichroic 630 (e.g. Cy5) from the "Mirror" dropdown menu , or simply choose the automatic mirror selection by clicking on "Automatic".

7. To set the measurement blank click on "Change" in the "Measurement" section and select the wells containing the buffer blank.

8. In the "G-Factor" section, select "Calibrate", and set the "Reference value" to 20 mP. To select the Reference Range, click on "Change" and select the wells containing the free tracer. To select the G-Factor blank range, click on "Same as measurement blank" to use the same wells selected in the "Measurement" section. If the plate is being read more than once, select "Manual" to use the G-Factor previously calculated by the instrument.

9. In the "Read" section, enter the number of flashes to be used, depending on the quality of data desired. The "Settle time" should be set at 0 ms.

10. In the "Gain" section, select "Optimal", to allow the instrument to calculate the proper gain based on each well to be read. If the plate is being read more than once, select "Manual gain" and enter the value that was previously calculated by the instrument.

11. In the "Z-Position" section, select "Calculated from well:" and select an appropriate well from the dropdown menu. If the plate is being read more than once, select "Manual" and enter the value previously calculated by the instrument.

12. In the "Label" section, choose Label1 from the "Name" dropdown menu, or simply type in a desired name for the measurement.

13. Select "Plate wise" in the second "Measurement" section to read all the parallel polarized intensities, followed by the perpendicular intensities, in order to decrease measurement time.

14. Click on the triangle at the bottom of the plate to "Show Details", and set the "Lag time" to 0 µs, and the "Integration time" to 20 µs.

Optimized Measurement Settings	
Plate Definition	Corning 384 Flat Bottom Black Polystyrol
Settle Time	0 ms
Z-Position	22969 µm (calculated from well A1)
Gain	94 (optimal)
Flashes	variable

Table 4. Instrument Settings

Fluorescence polarization measurements were performed using the settings listed in Table 4. The plate definition and settle time were manually selected, while all other settings were determined by the instrument. The number of flashes was varied manually 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 Infinite® F500 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.

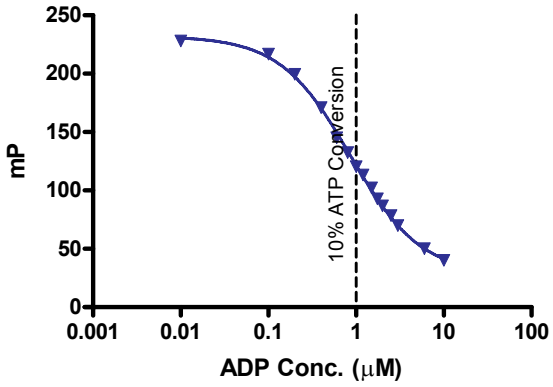


Figure 1. 10 µM ATP/ADP Standard Curve. 10% ATP Conversion represents 9 µM ATP/1 µM ADP concentration level.

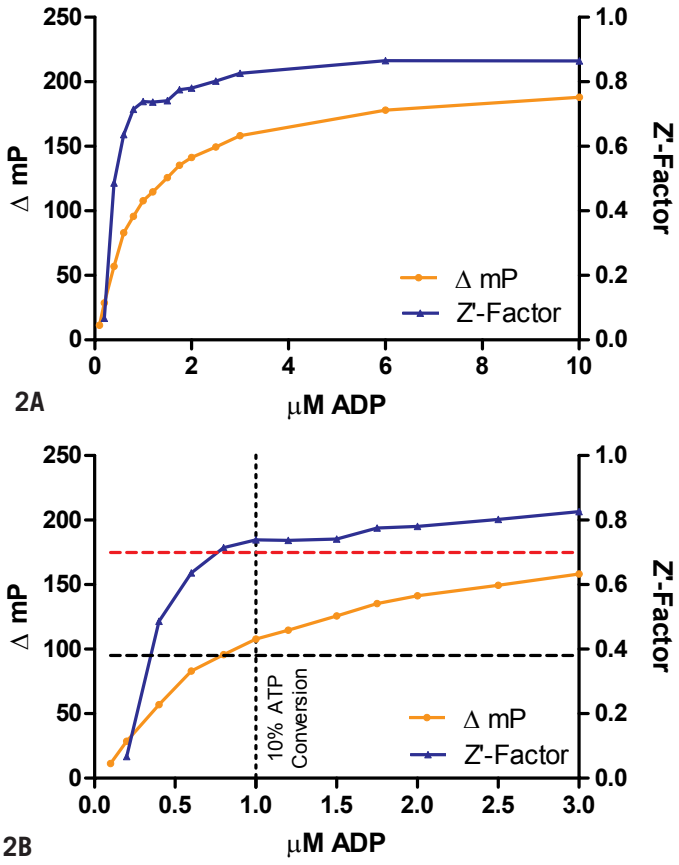


Figure 2. A) Z′ and Δ mP values observed in a standard curve mimicking conversion of 10 µM ATP to ADP. B) A zoom in of the 1-3 µM ADP section of the standard curve. Z′ validation minimal qualification shown by red dashed line. Δ mP validation minimal qualification shown by black dashed line. 10% ATP conversion validation point shown by black dotted line. Reader set at 3 flashes, and 0 ms settle time.

A Z′>0.7 and an mP shift of 108 units is achieved at 1.0 µM ADP (10% ATP conversion) in a read time of 2:22 (Figure 2A). The Z′ value falls below 0.7 at 6% substrate conversion, which coincides with a Δ mP value of 83. Figure 2B highlights data that is generated in the initial velocity range of the reaction. Validation criteria are met by the Infinite® F500 using a reader setting of 3 flashes and a 0 ms settle time.

Assay Performance at 10% Conversion of 10 µM ATP				
Flashes	**3**	**5**	**10**	**25**
Read Time (Minutes)	**2:22**	**3:03**	**4:41**	**7:20**
10% ATP Conversion ΔmP	108	109	108	109
10% ATP Conversion Std. Dev.	5	5	5	5
10% ATP Conversion Z′-Factor	0.74	0.78	0.76	0.76

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 3 flashes.

Discussion

The data shows that the Tecan Infinite® F500 Microplate Reader is compatible with the Transcreener Far Red FP Detection Module. Following the Transcreener protocol and setting the instrument to 3 flashes and a 0 ms settle time will yield Z′ values ≥ 0.7 in a read time of 2:22. The Z′ can be improved slightly by increasing the number of flashes at the expense of longer read times. 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

- Tecan Infinite® F500 Filter-Based Microplate Reader passed the validation criteria under the following conditions: Using a 610/20nm excitation filter, a 670/25nm emission filter, and a 630nm dichroic mirror; instrument was set to 3 flashes and a 0 ms settle time, and yielded a Z' >0.7 in 2:22 minutes

- Using optimized instrument settings, filters, and dichroic mirror recommended by Tecan reduces standard error in mP reads

Additional Information

Technical Information

For technical information, please contact Meera Kumar, Application Scientist-Biochemistry
608.443.2400
866.313.7881
meera.kumar@bellbrooklabs.com

Related Products

Transcreener® ADP² FP Assay 3010-1K
Transcreener® ADP² TR-FRET Red Assay 3011-1K
Transcreener® ADP² 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 Information

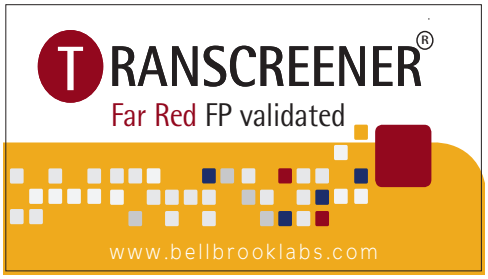
Please contact BellBrook Labs for product pricing. Custom quotes are available for orders of 10,000 wells or more.

Phone orders:
608.443.2400
866.313.7881

Fax orders:
608.441.2967

Email orders:
info@bellbrooklabs.com

Transcreener Instrument Validation Stickers



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