

In this guide:

- Recommended settings for the Molecular Devices Analyst® GT Multimode Microplate Reader
- Optimizing sensitivity setting and integration time improves data
- Analyst® GT 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.

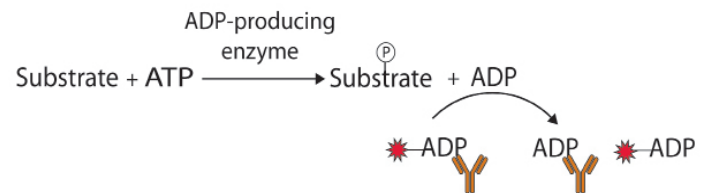
Assay Principle

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.

Transcreener® ADP Assay

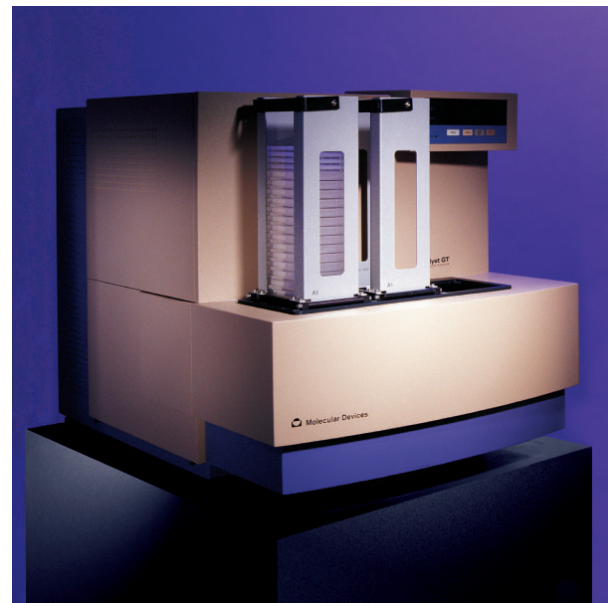
far red FP



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

Analyst® GT Information



- Standard 25mm filters for FP, TRF, TR-FRET, FI, and absorbance. Separate PMT for Luminescence.
- Readout maximization powered by 2nd generation Smart Optics® system.
- HTS compatible, high-speed bidirectional stacker option.
- Seamless integration into robotic environments.
- Plate formats supported: 96 to 1536 well microplates.

Materials and Methods

Instrument: Analyst® GT Multimode 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	
500 mM MgCl ₂	Not Provided
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 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 Analyst® GT Multimode 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 and Filter Information

Instrument Detection Components		Catalog #
Excitation Filter	620/35nm	42-000-0038 (MDS)
Emission Filter	682/22nm	XF3031 (Omega Optical)
Dichroic Mirror	650nm	0200-6120 (MDS)

Table 3. Instrument Optics

The AnalystHost application provides a graphical user interface for setting up the reader and running assays. To set up a Transcreener method in the AnalystHost application, go to the Methods menu and select Detection, then New. Choose Fluorescence Polarization in the dropdown menu and enter a name for the method. Click OK to view the Define and Edit Methods screen; the new method is assigned the default parameters for the selected method type. Continuous Lamp, Raw Data Units of Counts/sec, and Attenuator Mode Out should not be changed from their default settings. The continuous lamp is recommended for highest sensitivity in the fluorescence polarization detection mode.

If the microplate type being used is already represented by a microplate format in the Plate dropdown menu, select it here. Otherwise, use the Plates menu to define a new microplate format, entering microplate dimensions supplied by the manufacturer. From the Plates menu, select New. Enter the new plate name and number of wells. Click OK and enter microplate dimensions (in millimeters) in the resulting schematic representation of a microplate. Then click OK to save.

To customize the detection method for Transcreeper assays, enter a G-factor value of 1.4, and change the Z-height to 1 mm. Choose the appropriate filters from the dropdown menus (excitation 620-35, emission 682-22). The wavelength of the installed dichroic mirror (650 nm) will be displayed automatically.

Enter a Max Integration Time between 20,000 and 200,000 μ s, depending on desired read speed. A longer integration time may yield better Z factors. Set Target SD per well to 0 mP. All other parameters in the Define and Edit Methods screen should be left on the default values for Fluorescence Polarization. Click on the Advanced button to display the Advanced Fluorescence Polarization Setup screen. All parameters displayed here may be left as default; Plate settling time should be 10 ms.

Click Select Wells to choose wells to read. Up to eight Sample groups and corresponding Background groups can be assigned here. Row or Column read direction can also be selected.

Optimized Measurement Settings	
Microplate Format	User-defined or pre-defined format
Lamp	Continuous
Target SD per Well	Not Set
Attenuator Mode	Out
Raw Data Units	Counts/sec
Plate Settling Time	10 ms (in advanced setup)
Z Height	1 mm
Integration Time	variable

Table 4. Instrument Settings

Fluorescence polarization measurements were performed using the settings listed in Table 4. The G-factor and Z-height focus were manually adjusted, while all other settings were kept at the default settings. The integration time per well was manually adjusted in the AnalystHost Application. Integration time 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 $Z' \geq 0.5$ 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 Analyst® GT Multimode 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.

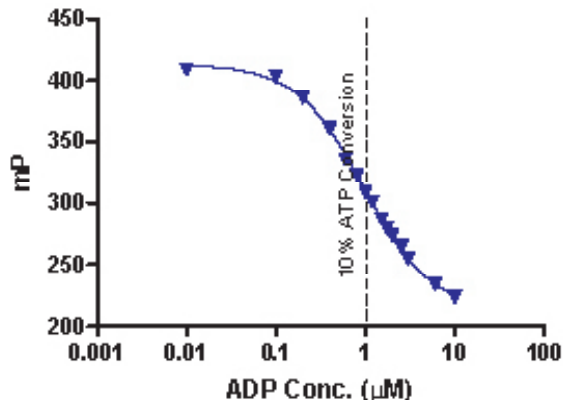


Figure 1. 10 μ M ATP/ADP Standard Curve. 10% ATP Conversion represents 9 μ M ATP/1 μ M ADP concentration level.

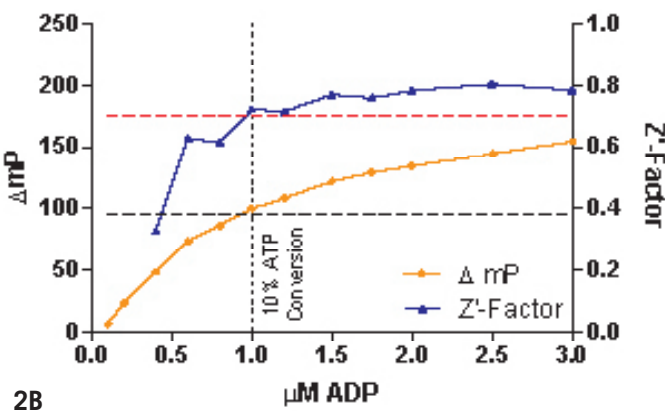
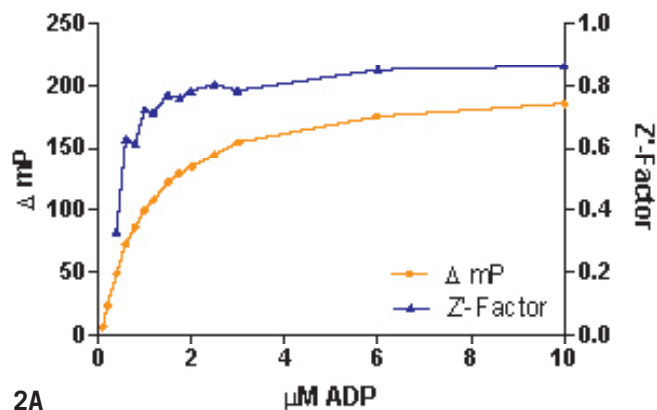


Figure 2. A) Z' and Δ mP values observed in a standard curve mimicking conversion of 10 μ M ATP to ADP. B) A zoom in of the 1-3 μ M ADP section of the standard curve. Z' validation minimal qualification shown by red dashed line. Δ mP validation minimal qualification shown by black dashed line. 10% ATP conversion validation point shown by black dotted line. Reader set at 20,000 μ s integration time.

A $Z' > 0.7$ and an mP shift of 100 units is achieved at 1.0 μ M ADP (10% ATP conversion) in a read time of 1:51 (Figure 2A). The Z' value falls below 0.7 at 8% substrate conversion, which coincides with a Δ mP value of 86. Figure 2B highlights data that is generated in the initial velocity range of the reaction. Validation criteria are met by the Analyst® GT using a reader setting of 20,000 μ s.

Assay Performance at 10% Conversion of 10 μ M ATP				
Integration Time (μ s)	10,000	20,000	30,000	50,000
Read Time (Minutes)	1:41	1:51	2:00	2:13
10% ATP Conversion Δ mP	102	100	100	99
10% ATP Conversion Std. Dev.	8	5	4	3
10% ATP Conversion Z'-Factor	0.53	0.72	0.76	0.80
Assay Performance at 10% Conversion of 10 μ M ATP				
Integration Time (μ s)	75,000	100,000	150,000	200,000
Read Time (Minutes)	2:36	2:52	3:36	4:17
10% ATP Conversion Δ mP	99	95	101	99
10% ATP Conversion Std. Dev.	2	3	3	3
10% ATP Conversion Z'-Factor	0.83	0.82	0.86	0.84

Table 5. Assay performance with various instrument settings.

Variable integration time was evaluated to determine the optimal read time generating the highest quality data. As integration time 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 a 20,000 μ s integration time.

Discussion

The data shows that the Molecular Devices Analyst® GT is compatible with the Transcreener Far Red FP Detection Module. Following the Transcreener protocol and setting the instrument to a 20,000 μ s integration time will yield Z' values \geq 0.7 in a read time of 1:51. The Z' can be improved 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 filters or dichroics may have adverse effects on instrument performance resulting in an increased standard error in reads.

Conclusions

- Molecular Devices Analyst® GT passed the validation criteria under the following conditions: Using a 620/35nm excitation filter, a 682/22nm emission filter, and a 650nm dichroic mirror; instrument was set to a 20,000 μ s integration time and yielded a Z' >0.7 in 1:51 minutes
- Using optimized instrument settings and the filters and dichroic recommended by Molecular Devices 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® ADP ² FP Assay.....	3010-1K
Transcreener® ADP ² TR-FRET Red Assay.....	3011-1K
Transcreener® ADP ² 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.

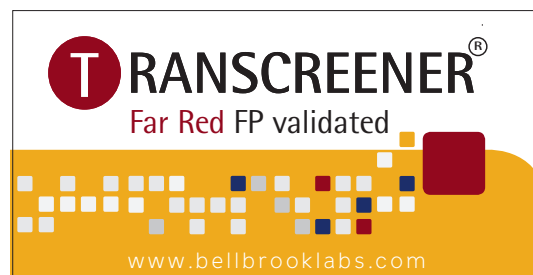
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Transcreener Instrument Validation Stickers



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