BellBrookLabs

Optimizing Performance of the Transcreener® ADP² TR-FRET Assays for the PerkinElmer EnVision® HTS Multilabel Plate Reader

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PerkinElmer instruments

Application Note

In this guide:

Setting recommendations using the PerkinElmer EnVision® for the Red TR-FRET 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.

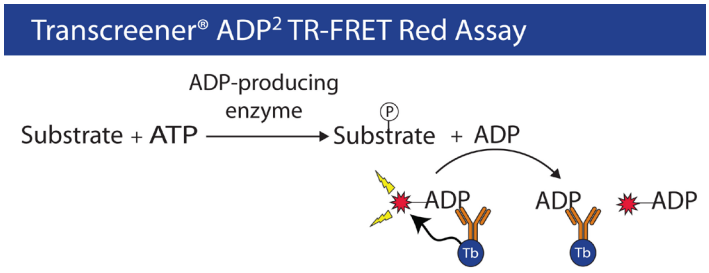
Sensitivity
High affinity antibodies enable robust detection of low levels of substrate conversion (<10%) with less enzyme than other methods.

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, monochromater settings and read times impact an instrument's sensitivity with any given assay. 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® ADP² TR-FRET Red Assay is a competitive immunoassay for ADP with a far-red, time-resolved Förster-resonance-energy-transfer (TR-FRET) readout. The Transcreener ADP Detection Mixture comprises an ADP HiLyte647 Tracer bound to an ADP² Antibody-Tb conjugate. Excitation of the terbium complex in the UV range (ca. 330 nm) results in energy transfer to the tracer and emission at a higher wavelength (665nm) after a time delay. ADP produced by the target enzyme displaces the tracer which causes a decrease in TR-FRET. The time gated nature of the detection method largely eliminates interference that can result from prompt fluorescence of test compounds. Use of a far red tracer further minimizes interference from fluorescent compounds and light scattering.

Transcreener® ADP² TR-FRET Red 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 (Table 2). Instrument settings were optimized to read the results of the standard curves. Because individual results may vary, recommended settings 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**
- **Interchangable filters and dichroic modules**

Materials

Instrument: EnVision® 2100 Multilabel Plate Reader

Microplates: Corning® 384 Well Low Volume White Round Bottom PS NBS™ Microplate (Product #3673)

Reagent	Kit/Component Catalog #
Transcreener® ADP² TR-FRET Red Assay	3011-1K
ADP HiLyte647 Tracer	2060
Stop & Detect Buffer C, 10X	2062
ADP² Antibody-Terbium Conjugate, 800 nM	2061
5 mM ADP	2052
5 mM ATP	2053
Buffer Components	
500 mM EGTA	Not Provided
1000 mM HEPES	
500 mM $MgCl_2$	
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 $MgCl_2$, 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 C, 8 nM ADP² Antibody-Tb, and 27nM of ADP HiLyte647 Tracer.

High FRET Mixture

High FRET mixture consistes of 8nM ADP² Antibody-Tb, 27nM of ADP HiLyte647 Tracer, 10 µM of ATP in 1X Stop & Detect Buffer C.

Low FRET Mixture

Low FRET mixture consistes of 8nM ADP² Antibody-Tb, 27nM of ADP HiLyte647 Tracer, 10 µM of ADP in 1X Stop & Detect Buffer C.

Final Concentrations in 20 uL 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 C (25 mM HEPES, pH 7.5, 10 mM EDTA, and 0.01% Brij-35), 4 nM ADP² Antibody-Tb, and 13nM of ADP HiLyte647 Tracer.

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 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 high FRET mixture was dispensed to wells P1-P12, and 10 µL of low FRET mixture was dispensed to wells P13-P24.

Table 2. Standard Curve ATP/ADP Concentrations

Substrate Conversion Levels (%)	ATP, µM	ADP, µM
0	10	0
0.1	9.99	0.01
0.25	9.975	0.025
0.5	9.95	0.05
1	9.90	0.1
2.5	9.75	0.25
5	9.5	0.5
7.5	9.25	0.75
10	9.0	1.0
15	8.5	1.5
20	8	2
30	7	3
50	5	5
70	3	7
100	0	10

Table 3. Instrument Optics

Instrument Set-up and Filter Information

Ex. Filter/ Em. Filter		Perkin Elmer Catalog #
Excitation Filter	320/7.5 nm	2100-5060
Emission Filter-1	615/8.5 nm	2100-5090
Emission Filter-2	665/7.5nm	2100-5110
Mirror	Lance Dual D400/ D630	2100-4160

The following steps were taken to prepare the EnVision to correctly read the Transcreener® ADP² TR-FRET Assay:

1. A label was created by replicating an existing label (Lance TRF under Wallac protocols).
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"and "Measurement Height"were chosen to be optimized.
5. Following completion of the Wizard, the measurement height was recorded from the "Optimization" tab for the label. The optimization was then deleted. The 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	
Window time	100µs
Measurement Height	11.6mm
Delay	60µs
Flash Number	variable
Flash number with second detector	20
Time between flashes	2000µs
Excitation light	100%

Table 4. Instrument Settings

TR-FRET measurements were performed using the settings listed in Table 4. The Measurement Height was optimized prior to reading the plate, using the procedure previously described. The number of flashes per well 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

Z'-Factor Calculation

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

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 free tracer increases resulting in an overall decrease in FRET.

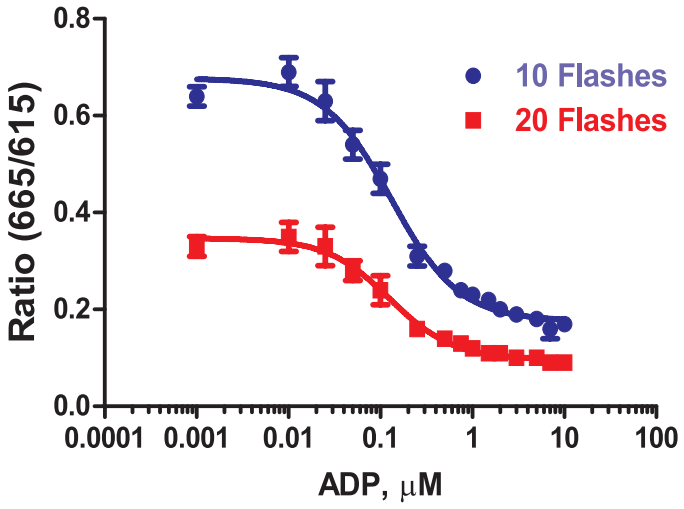


Figure 1. 10 µM ATP/ADP Standard Curve. 10% ATP Conversion represents 9 µM ATP/1 µM ADP concentration level.

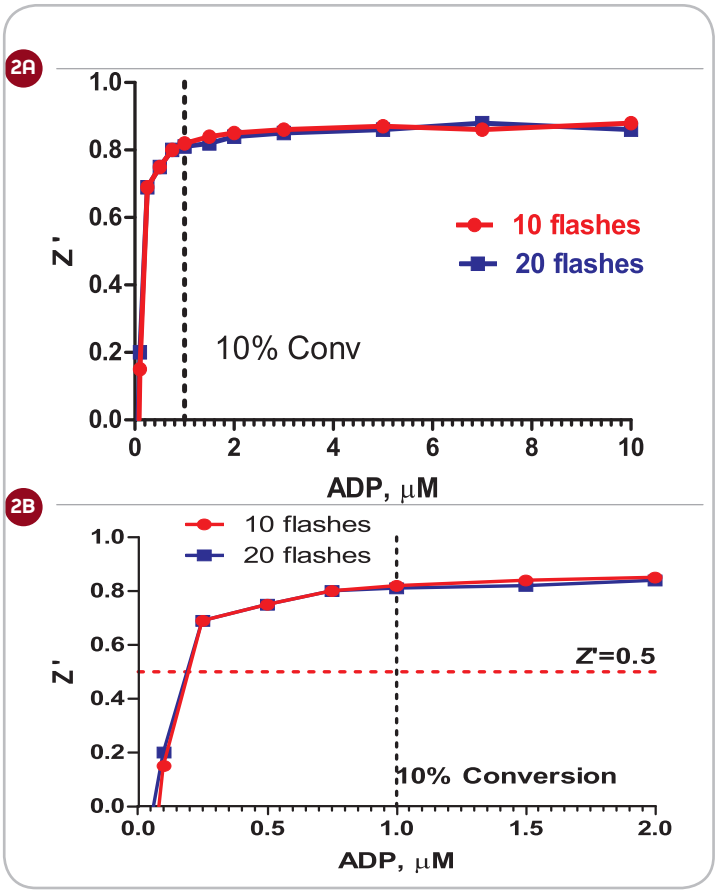


Figure 2. A) Z' observed in a standard curve mimicking conversion of 10 µM ATP to ADP. B) A zoom in of the 0-2 µM ADP section of the standard curve. Z' validation minimal qualification shown by red dashed line. 10% ATP conversion validation point shown by black dotted line. Reader set at 10 flashes or 20 flashes.

A Z' > 0.7 is achieved at 1.0 µM ADP (10% ATP conversion) in a read time of 4:34 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 PerkinElmer EnVision® using a reader setting of 10 flashes.

Table 5. Assay Performance at Various Instrument Settings

Assay Performance at 10% Conversion of 10 µM ATP					
Flashes	**1**	**5**	**10**	**20**	**100**
Read Time (Minutes)	**1:27**	**2:22**	**4:34**	**5:06**	**7:24**
% CV at 10% ATP Conversion	**8.85**	**4.91**	**3.66**	**3.32**	**3.16**
Z'-Factor at 10% ATP Conversion	**0.51**	**0.65**	**0.82**	**0.81**	**0.84**

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

Discussion

The data shows that the Transcreener ADP² TR-FRET Red 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 10 flashes yielded Z′ values ≥ 0.7 in a read time of 4:34 minutes. The data also shows that the Transcreener ADP² TR-FRET Red 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

PerkinElmer's EnVision® Multilabel Plate Reader met the performance criteria under the following conditions: Lance Dual D400/D630 mirror with 320/7.5nm excitation and emission filters of 615/8.5 nm and 665/7.5nm **combined with 10 instrument flashes with a postion delay of 60µs and window time of 100µs yielded a Z′ >0.7 in 4:34 min.**

Using the optimized instrument setup recommended by PerkinElmer reduces standard error in FRET measurements.

Transcreener ADP² TR-FRET assay improves data quality using initial rate enzyme reaction kinetics.

Additional Information

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

Technical Information

For technical information, please contact
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Ordering

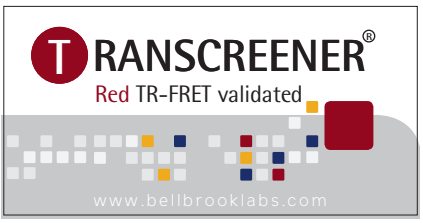
Please contact BellBrook Labs for product pricing.
Custom quotes are available for orders of 10,000 wells or more.

Phone orders:
608 • 443 • 2400
866 • 313 • 7881

Fax orders:
608 • 441 • 2967

Email orders:
info@bellbrooklabs.com

Transcreener Instrument Validation Stickers



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