Utility of The Synergy™ H1 Multi-Mode Microplate Reader in Combination with Transcreener® ADP² FP, TR-FRET and FI Assays for The Measurement of ADP Accumulation

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Overview

- The sensitivity of the Transcreener® ADP² Assays provides the capability to detect ADP formation using initial rate kinetics.
- The Synergy™ H1 Multi-Mode Reader combines two fluorescence detection systems, monochromator-based and filter-based, into one unit.
- The instrument's deep blocking filters and dichroic mirrors provide quality data using short read times with all fluorescence detection modes.
- The monochromator system allows multiple fluors to be tested during assay development without the need to switch out filters.
- Data generated with the Transcreener ADP² Assay, as well as the results of the spectral scan, demonstrate the ease that the assay's detection modules can be read, as well as the ability of the reader to yield high quality information using either detection system.

Introduction

ADP formation represents an important way to monitor the activity of kinases and other ATP-utilizing enzymes. Kinases, which produce ADP by transferring the terminal phosphate group from ATP to a substrate, remain one of the most highly screened target classes today, due to their role in a wide range of diseases, including many types of cancer. ATPase enzymes, including heat shock protein 90 (Hsp90) and Hsp72 are also emerging as potential drug targets for cancer research due to their role in stabilizing mutant proteins which may contribute to carcinogenesis. The ability to monitor these reactions using initial rate kinetics signifies that a low turnover of ATP to ADP will be exhibited. Therefore an assay that is sensitive enough to distinguish between these subtle changes, coupled with a microplate reader which can detect the small differences in signal, are necessary.

Here we demonstrate the combination of a variety of fluorescence-based assays and a multi-mode reader to be used for the detection of ADP using initial rate kinetics. The Transcreener ADP² Assay is based on the immunodetection of ADP, and has been developed to utilize three of the most widely accepted detection technologies currently in use today, including Fluorescence Polarization (FP), Fluorescence Intensity (FI), and Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET). The instrument is a hybrid multi-mode microplate reader, which combines a monochromator-based as well as a filter-based detection system. The filter module, which was used in this instance, is completely independent of the monochromator system, and includes its own light source and dichroic-based wavelength detection system. A 10 µM ADP/ATP standard curve was set up using increasing and decreasing concentrations of ADP and ATP, respectively, keeping the total adenine concentration constant in each well. The curve mimics the conversion of ATP to ADP in an enzyme reaction. Z'-Factor values and relative standard deviation were determined using the 10 µM ATP/0 µM ADP and 9 µM ATP/1 µM ADP points on the curve. The data generated demonstrate the ability of assay and reader to be used together to detect the formation of ADP by these important drug targets.

Synergy™ H1

The Synergy™ H1 combines a filter-based and monochromator-based fluorescence detection system in the same unit. The monochromator system allows the unit to be used during assay development, where multiple known and unknown wavelengths may be tested. The filter module gives the instrument the sensitivity needed to run all major fluorescence detection modes, as well as filtered luminescence (e.g. BRET).

The monochromator system uses two double-grating monochromators. The quadruple design provides continuous wavelength selection from 230-999 nm, as well as the highest stray light rejection, and spectral and well-area scanning capabilities.

The filter module delivers more energy to the sample and provides high signal-to-noise ratios. The incorporation of deep blocking filters and dichroic mirrors into the same compact unit provides faster read speed, more sensitivity, and more precise control over optical parameters for fluorescence, time-resolved fluorescence resonance energy transfer (TR-FRET), and fluorescence polarization applications. It also provides a simple, efficient way to change optics when necessary.

Figure 1 – Synergy H1 Hybrid Multi-Mode Microplate Reader.

Transcreener ADP² Assay

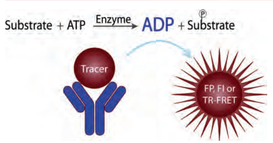

Figure 2 – Addition of ADP causes the displacement of the tracer from the appropriate antibody. The amount of free tracer detected is proportional to the concentration of ADP within the well.

Experimental Design

- 15-point standard curves were set up containing various combinations of ATP and ADP, ranging from 10 µM ATP/0 µM ADP to 0 µM ATP/10 µM ADP. A constant concentration of 10 µM Adenine was maintained at each point included on the curve.
  - The concentrations of ATP and ADP at each point on the curve mimic the conversion of ATP to ADP in an enzyme reaction.
  - Two points on the curve were used for assay quality measurement. 10 µM ATP/0 µM ADP (100% ATP/0% ADP or 0% ATP Conversion) and 9 µM ATP/1 µM ADP (90% ATP/10% ADP or 10% ATP Conversion).
- Twenty-four 10 µL replicates of each point on the curve were added to a Corning 384-Well Low-Volume assay plate.
- The appropriate assay detection mixture was then added to each plate at a 10 µL volume.
- The plates were mixed for 30 seconds on an orbital shaker, covered, and incubated for 1 hour at room temperature, and then read on the Synergy H1.
- Two data quality measurements were created with each test.
  - Precision - %CV (Assessed using 10% ATP conversion std. curve point only)
  - Assay Robustness – Z'-Factor[1] (Assessed using both std. curve points)

Fluorescence Polarization Assay Capabilities

Transcreener® ADP² FP Assay far red FP

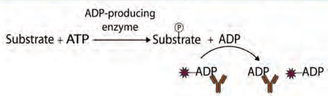

Figure 3 – The Transcreener ADP Detection Mixture comprises an ADP Alexa633 Tracer bound to an ADP² antibody. The tracer is displaced by ADP, the invariant product generated during the enzyme reaction. The displaced tracer freely rotates leading to a decrease in fluorescence polarization. Parallel and perpendicular signals were read on the Synergy H1 using 620/40nm and 680/30nm excitation and emission filters, along with a 660nm cutoff dichroic mirror.

Test – The affect of increasing read time on Transcreener ADP² FP Assay data quality was assessed using the Xenon lamp, along with variable flashes.

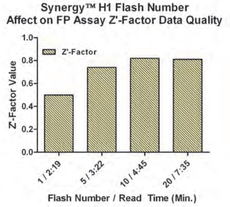

Figure 4 – FP Assay: Flash Number Effect on Z'-Factor Data Quality. Excellent Z' values can be achieved using as little as 5 flashes. Therefore high data quality can be seen while maintaining high-throughput.

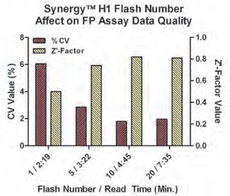

Figure 5 – FP Assay: Flash Number Effect on Data Variability and Quality. Additional flashes decrease the variability within measurements. Therefore, due to the Z'-Factor equation, this phenomenon increases the final Z'-Factor score for that data set.

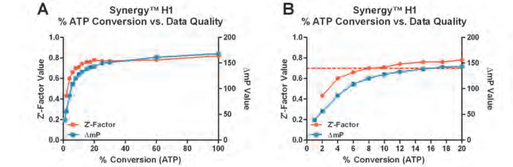

Figure 6 – FP Assay: Effect of ATP Conversion on Assay Quality. A. Assay window and Z'-Factor scores improve with increasing ATP conversion. B. Excellent Z'-Factor scores are able to be achieved with as little as 8% conversion of ATP to ADP.

Fluorescence Intensity Assay Capabilities

Transcreener® ADP² FI Assay

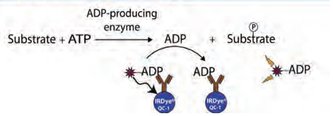

Figure 7 – The Transcreener ADP Detection Mixture comprises a quenched ADP Alexa594 Tracer bound to the ADP² monoclonal antibody conjugated to an IRDye QC-1 quencher. The tracer is displaced by ADP, the invariant product generated during an enzyme reaction. The displaced tracer becomes un-quenched in solution leading to a positive increase in fluorescence intensity. The assay was read by the filter module using a 560/40nm and 635/32nm excitation and emission filter, along with a 595nm cutoff dichroic mirror.

Test – The affect of increasing read time on Transcreener ADP² FI Assay data quality was assessed using the Xenon lamp, along with variable flashes.

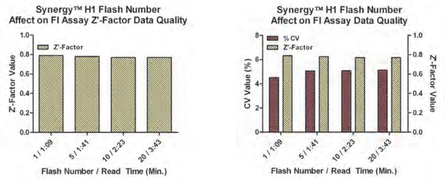

Figure 8 – FI Assay: Flash Number Effect on Data Variability and Quality. Low variability and excellent Z' values can be achieved using as little as 1 flash. Therefore high quality data is achieved no matter what read speed is chosen.

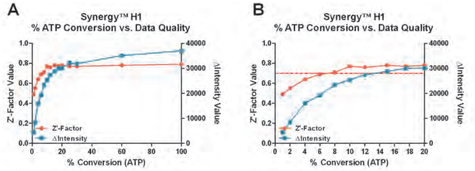

Figure 9 – FI Assay: Effect of ATP Conversion on Assay Quality. A. Assay window and Z'-Factor scores also improve with increasing ATP conversion. B. Excellent Z'-Factor scores are able to be achieved with as little as 6% conversion of ATP to ADP.

TR-FRET Assay

Transcreener® ADP² TR-FRET Red Assay

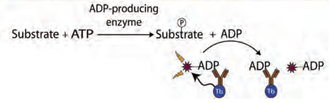

Figure 10 – The Transcreener ADP Detection Mixture comprises an ADP HiLyte647 Tracer bound to an ADP² Antibody-Tb conjugate. Excitation of the terbium complex in the UV range (ca. 330 nm) results in energy transfer to the tracer and emission at a higher wavelength (665nm) after a time delay. ADP produced by the target enzyme displaces the tracer which causes a decrease in TR-FRET, and emission at 620nm. The assay was read using a 340/30nm excitation filter, and 620/10 and 665/7.5nm emission filters, along with a 400nm cutoff dichroic mirror.

TR-FRET Assay Capabilities

Test – The affect of increasing read time on Transcreener ADP² TR-FRET Assay data quality was assessed using the Xenon lamp, along with variable flashes.

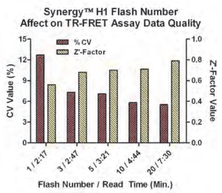

Figure 11 – TR-FRET Assay: Flash Number Effect on Z'-Factor Data Quality. Low variability within replicates, and excellent Z' values can be achieved using as little as 5 flashes. This once again demonstrates that high throughput can be achieved, while still maintaining high quality data.

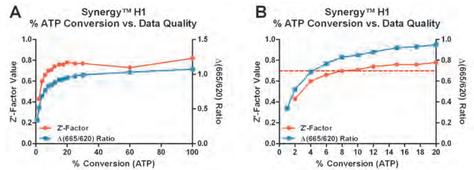

Figure 12 – TR-FRET Assay: Effect of ATP Conversion on Assay Quality. A. Assay window and Z'-Factor scores improve using the TR-FRET assay with increasing ATP conversion. B. Excellent Z'-Factor scores are able to be achieved with as little as 8% conversion of ATP to ADP.

Spectral Scanning Capabilities

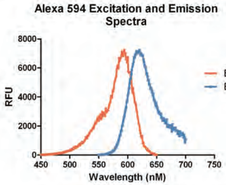

Figure 13 – Spectral Scanning Experiment. An excitation and emission spectral scan of the Alexa594 Tracer from the Transcreener ADP² FI Assay was performed using the monochromator system on the Synergy H1. Excitation scans were performed from 450-650nm in 1nm increments, with emission set at 680nm. Emission scans were performed from 550-700nm in 1nm increments, with excitation set at 520nm.

Conclusions

1. The Transcreener ADP² Assays are sensitive enough to distinguish between subtle increases in ADP concentration seen using initial rate kinetics.
2. The filter system on the Synergy H1 provides excellent data quality with ATP conversion rates less than 10%, using read speeds that maintain high-throughput.
3. The monochromator system of the Synergy H1 provides excellent spectral scanning capabilities.

1. Zhang et al.: A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *Journal of Biomolecular Screening* 1999 4(2):67-73.