

Utility of Hybrid Multi-Mode Readers using Both Monochromator- and Filter-Based Detection Capabilities as Demonstrated with Fluorescence Polarization, Time-Resolved Fluorescence Resonance Energy Transfer, and Fluorescence Intensity Assays for the Measurement of ADP Accumulation

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Overview

- The Synergy™ H4 Hybrid Microplate Reader incorporates BioTek's patent-pending Hybrid Technology™ which combines two fluorescence detection systems, monochromator-based and filter-based, into one unit.
- The monochromator system allows multiple fluors to be tested during assay development without the need to switch out filters. Fluors requiring higher spectral resolution, or possessing narrow Stokes shifts can easily be scanned using the variable (9-20 nm) bandpass capability in the system.
- The sensitivity of the instrument's deep blocking filters and dichroic mirrors provide quality data using short read times with all fluorescent detection modes.
- 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

Multi-mode microplate readers are one of the more indispensable laboratory instruments used in life science research today. Advances in reader technology now allow for an incredible array of assays to be performed with readout based on the detection of a wide range of fluorophores, luminophores, and chromophores with spectral properties in the UV to IR. Most readers are either monochromator- or filter-based. Monochromators offer the flexibility to read a virtually unlimited range of excitation and emission wavelengths, without the additional expense and hassle of purchasing and swapping out a wide range of filters during the assay development phase. Monochromators also provide the ability to determine the spectra of chromophores and fluorophores for multiple applications including UV-Vis spectra of protein folding and assessing purity of isolated biomolecules. Filters provide increased sensitivity and faster read speeds for higher throughput, miniaturized assays. While each type of reader has advantages, the capabilities of the other detection system are sacrificed when purchasing one or the other.

Here we show the utility of a hybrid multi-mode reader that combines the flexibility of monochromators with the sensitivity and speed of filter-based detection. Two double-grating monochromators are used to provide the highest stray light rejection, and continuous wavelength selection. Deep blocking filters and dichroic mirrors deliver high photon flux to the microplate wells and provide high signal-to-noise ratios in assays. We used three fluorescence-based assay technologies to test the capabilities of each detection system. The assays utilize Fluorescence Polarization, Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET), or Fluorescence Intensity for the measurement of ADP accumulation. 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. Scans of the ADP Alexa594 Tracer used in the Fluorescence Intensity assay were also performed. The data generated, as well as the results of the spectral scan, demonstrate the ability of the reader to yield high quality information using either detection system and its utility in today's life science research laboratory.

Synergy™ H4



Figure 1 – Synergy™ H4 Hybrid Multi-Mode Microplate Reader

The Synergy™ H4 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 system gives the instrument the sensitivity needed to run all major fluorescence detection modes, including AlphaScreen®/AlphaLISA®.

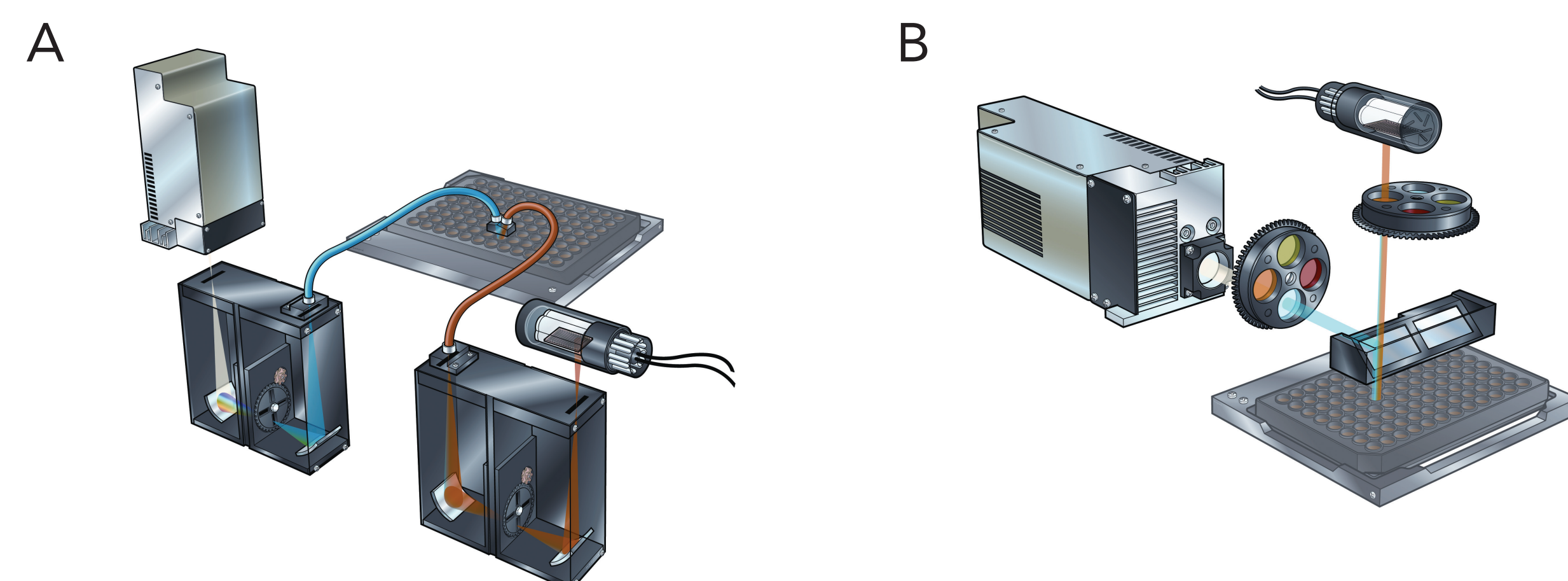


Figure 2 – Synergy™ H4 optical system A. Monochromator system; B. Filter system

The Synergy™ H4 monochromator system uses two double-grating monochromators with variable bandpass selection. The quadruple design provides continuous wavelength selection from 250-900 nm, as well as the highest stray light rejection, and spectral scanning performance.

The Synergy™ H4 filter/mirror system delivers more energy to the sample and provides high signal-to-noise ratios. The incorporation of deep blocking filters and dichroic mirrors provide faster read speed, more sensitivity, more precise control over optical parameters for fluorescence, time-resolved fluorescence resonance energy transfer (TR-FRET), and fluorescence polarization applications.

Experimental Design

- 15-Point Standard curves were setup 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 RT, and then read on the Synergy™ H4.
- Two data quality measurements were created with each test.
 - Precision - %CV (Assessed using 10% ATP conversion std. curve point only)
 - Assay Robustness – Z'-Factor (Assessed using both std. curve points)

Fluorescence Polarization Assay Capabilities

Transcreener® ADP² FP Assay

far red FP

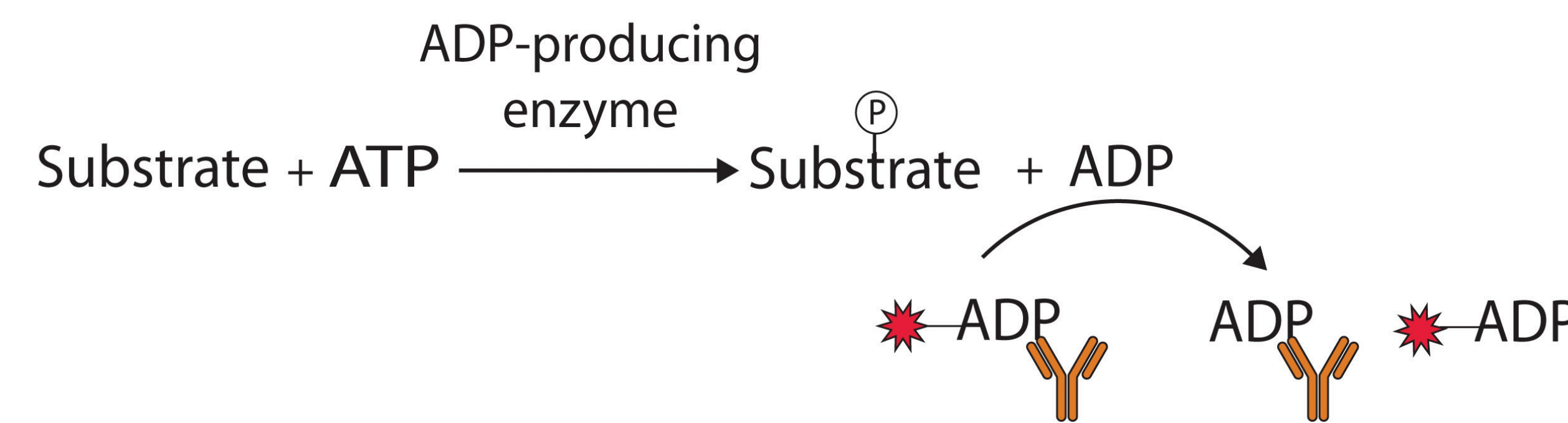


Figure 3 – The Transcreener® ADP detection mixture comprises an ADP Alexa633 Tracer bound to an ADP2 Antibody. The tracer is displaced by ADP, the invariant product generated during the enzyme reaction. The displaced tracer freely rotates leading to a decrease in fluorescence polarization. Parallel and perpendicular signals were read on the Synergy™ H4 using 620/40 nm and 680/30 nm excitation and emission filters, along with a 660 nm cutoff dichroic mirror.

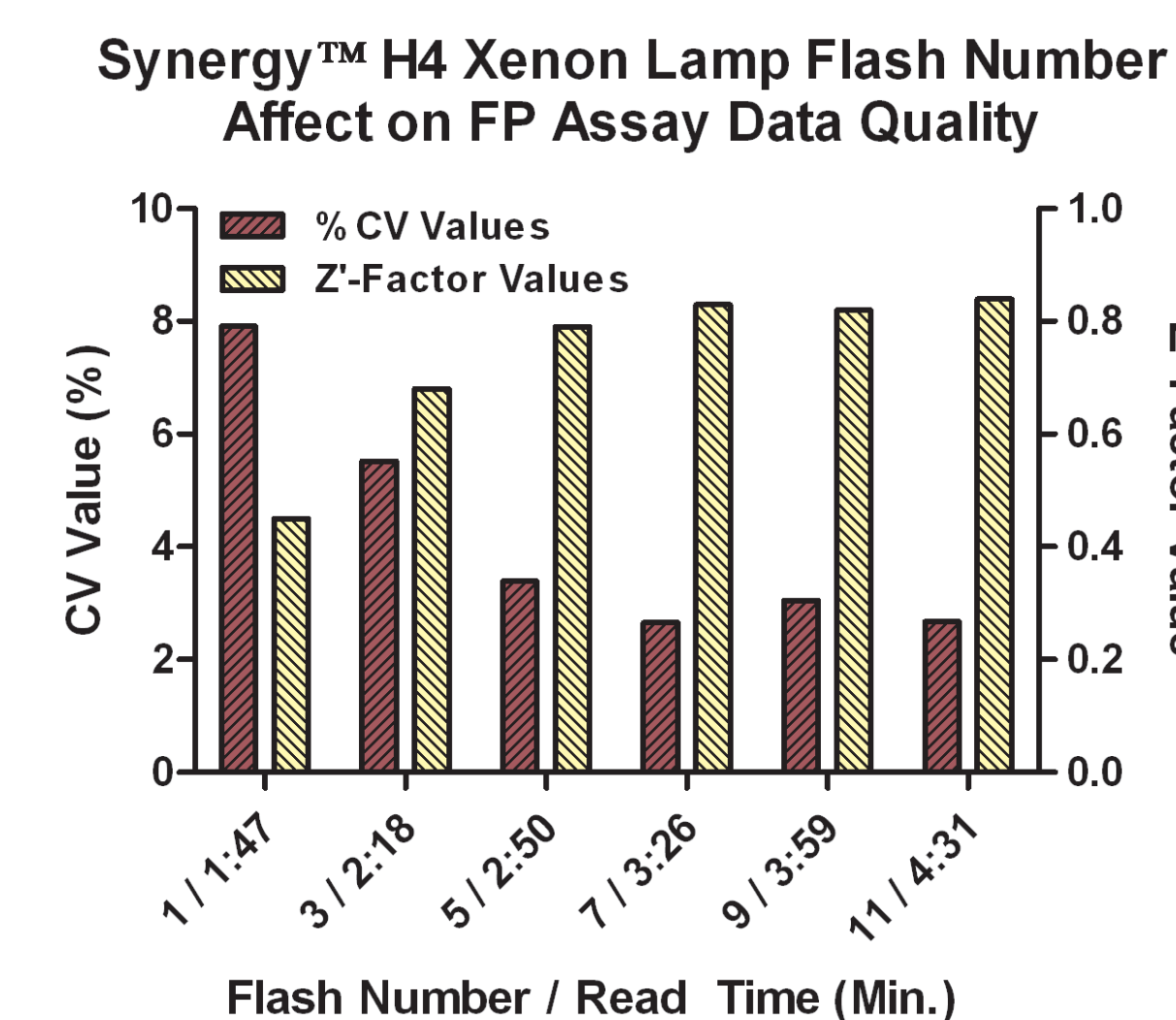


Figure 4 – FP assay experiment 1

Test – The Xenon lamp was used, along with variable flashes, to assess the affect of increasing read time on data quality, with a 660 nm cutoff dichroic mirror.

Summary – Excellent Z' values can be achieved using as little as 5 flashes. Therefore high data quality can be seen while maintaining high-throughput.

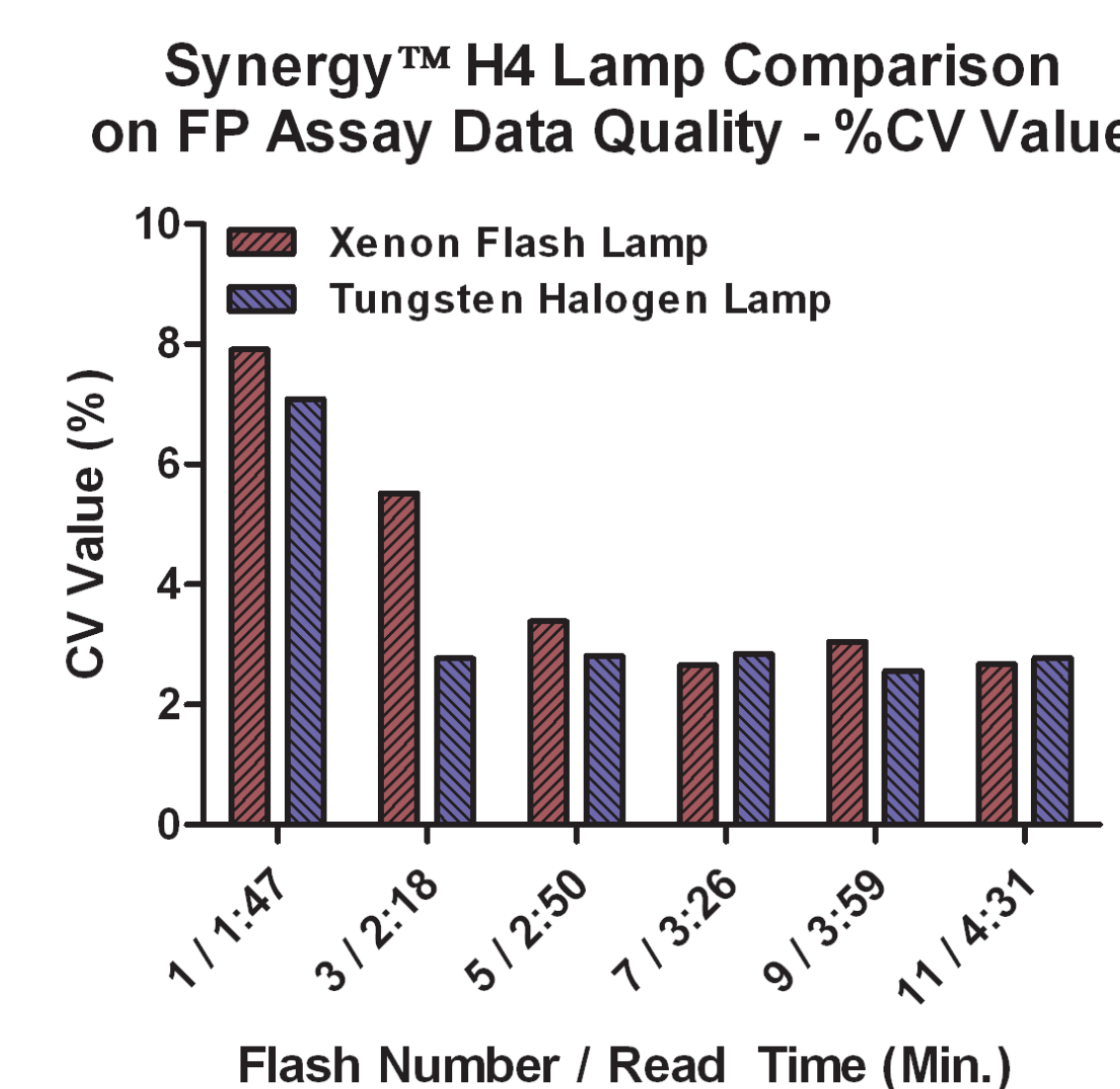


Figure 5 – FP assay experiment 2

Test – The Tungsten lamp was then tested, along with variable flashes, to compare data quality between the two lamps.

Summary – The Tungsten lamp provides slightly better data quality at lower flash numbers, whereas the lamps perform equally well at 5 flashes or above.

Fluorescence Intensity Assay Capabilities

Transcreener® ADP² FI Assay

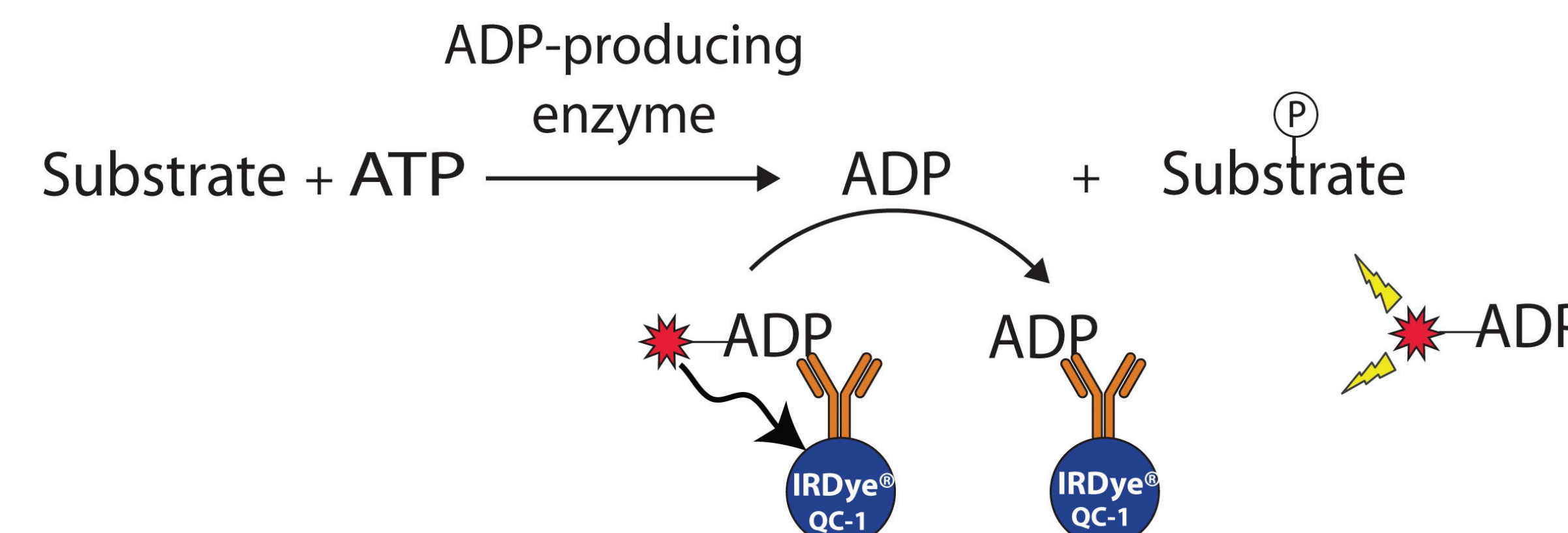


Figure 6 – The Transcreener® ADP Detection Mixture comprises a quenched ADP Alexa594 Tracer bound to the ADP2 monoclonal antibody conjugated to an IRDye QC-1 quencher. The tracer is displaced by ADP, the invariant product generated during an enzyme reaction. The displaced tracer becomes un-quenched in solution leading to a positive increase in fluorescence intensity. The assay was read by the filter system using a 590/20 nm and 620/10 nm excitation and emission filter, along with a 595 nm cutoff dichroic mirror. The assay was read by the monochromator system by setting excitation to 575 or 590 nm, setting emission to 620 nm, and using the variable bandpass feature.

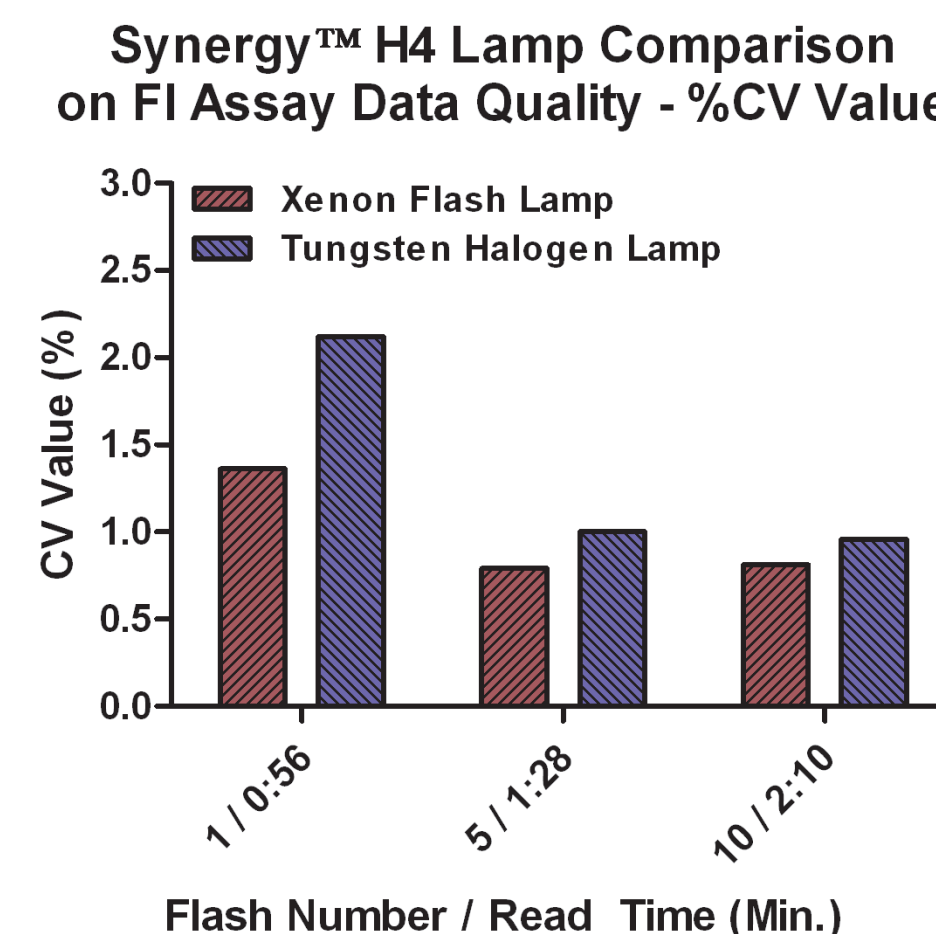


Figure 7 – FI assay experiment 1

Test – The Xenon and Tungsten lamps were tested, along with variable flashes, to compare data quality.

Summary – Excellent Z' values can be achieved using as little as 1 flash. Therefore high data quality can be seen at the fastest read speeds.

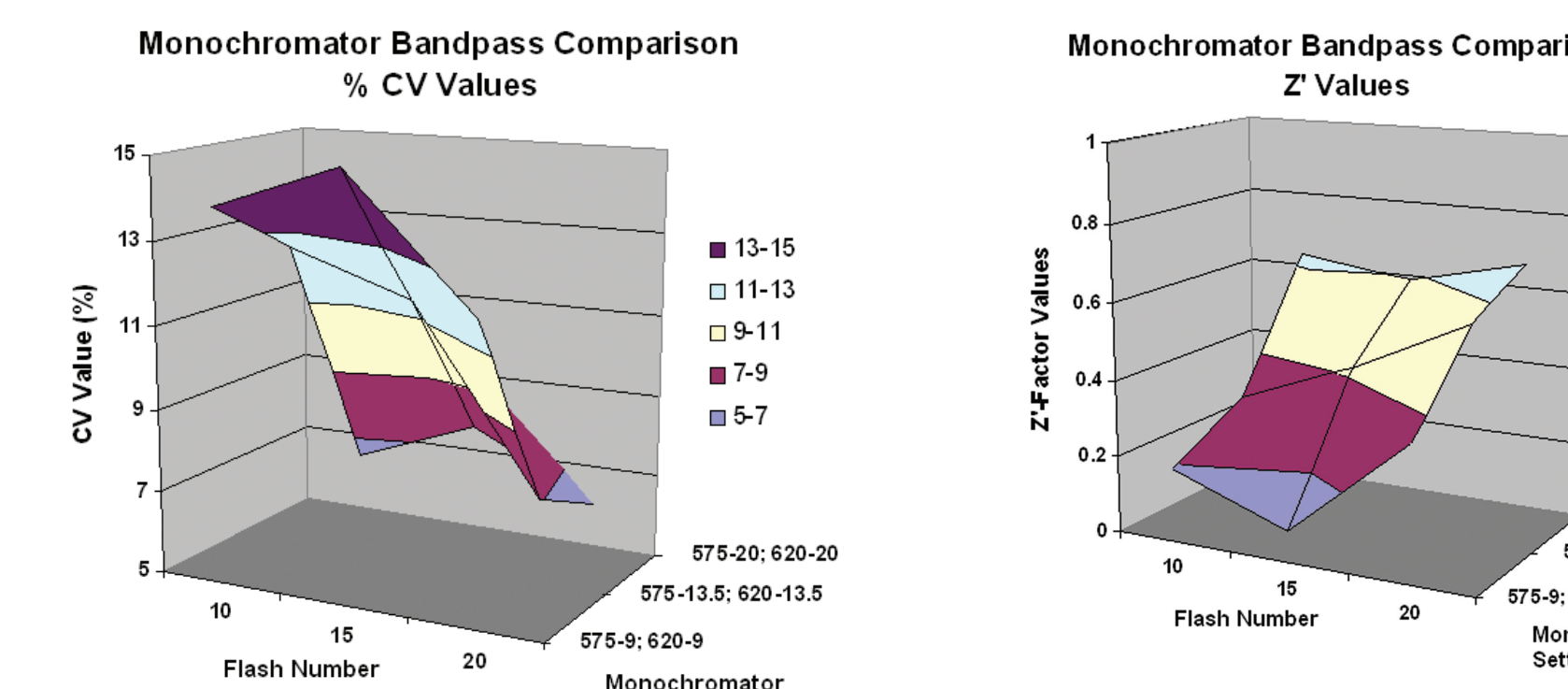


Figure 8 – FI assay experiment 2

Test – The monochromator system was tested using the Xenon lamp, along with variable flashes and bandpass settings, to compare data quality.

Summary – At each read speed (flash number), data quality can be improved by using wider monochromator bandpass settings.

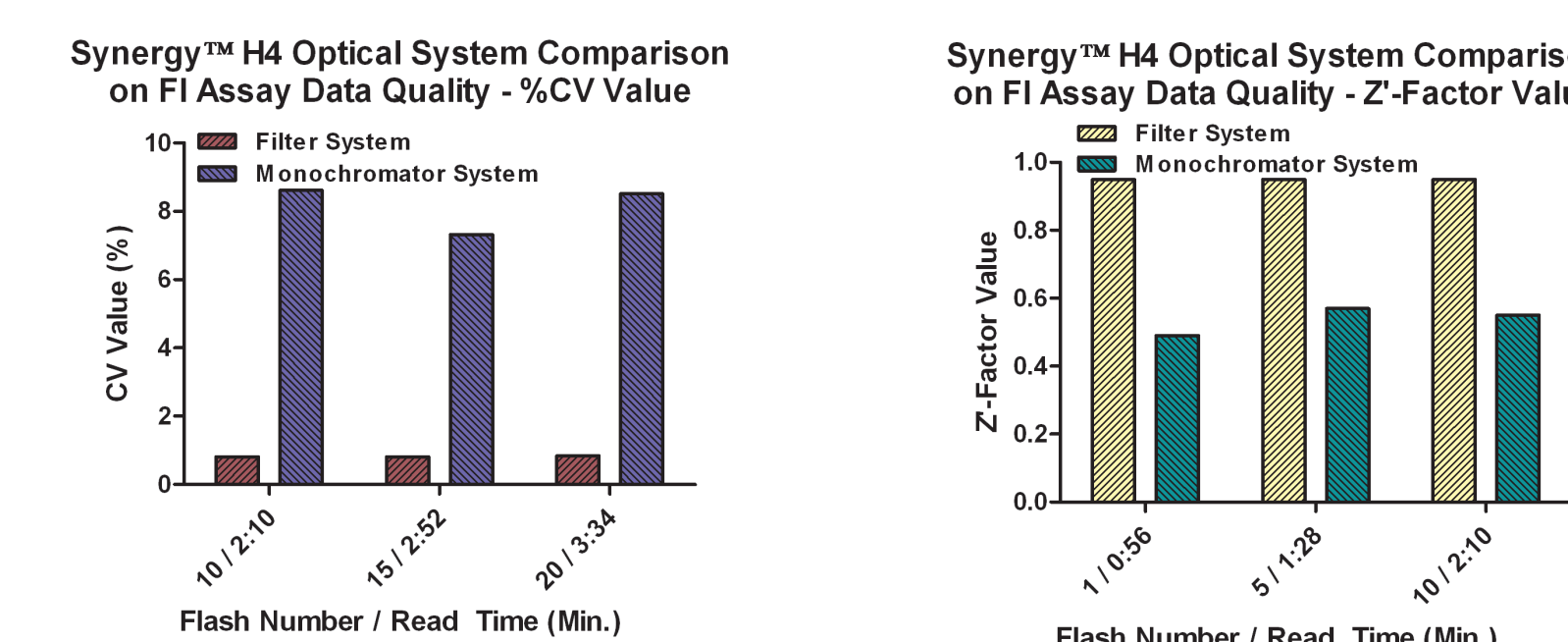


Figure 9 – FI assay experiment 3

Test – Data quality with the filter and monochromator systems was compared using equivalent read times.

Summary – At each read speed (flash number), data quality from the filter system exceeds that of the monochromator system. Therefore when performing applications where the highest quality data is necessary, use of the filter system is recommended.

TR-FRET Assay Capabilities

Transcreener® ADP² TR-FRET Red Assay

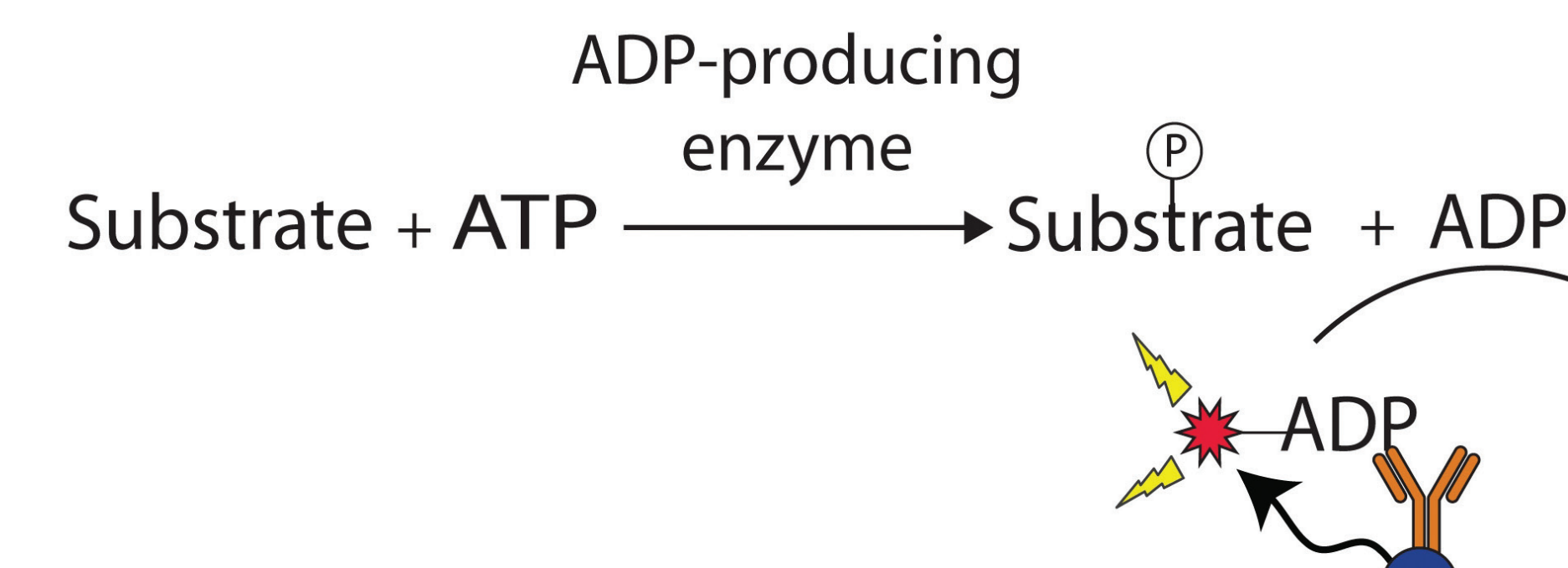


Figure 10 – The Transcreener® ADP Detection Mixture comprises an ADP HiLyte647 Tracer bound to an ADP2 Antibody-Tb conjugate. Excitation of the terbium complex in the UV range (ca. 330 nm) results in energy transfer to the tracer and emission at a higher wavelength (665 nm) after a time delay. ADP produced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

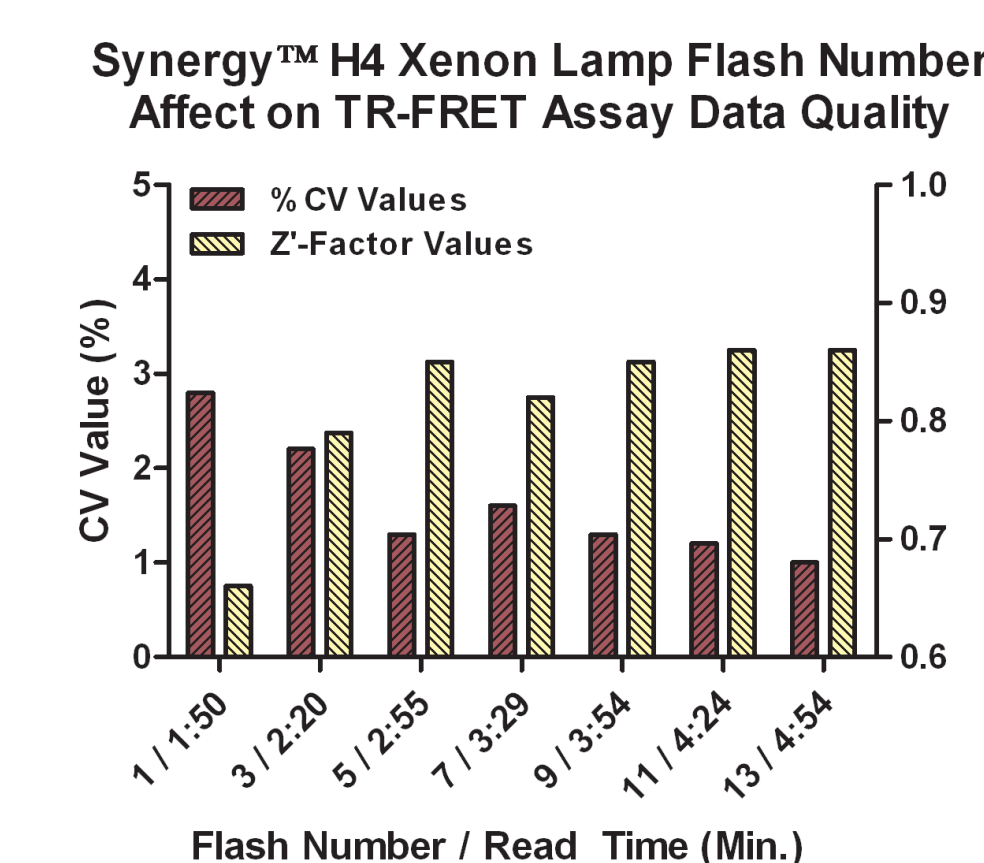


Figure 11 – TR-FRET assay experiment 1

Test – The Xenon lamp was used, along with variable flashes, to assess the affect of increasing read time on data quality.

Summary – Excellent Z' values can be achieved using as little as 3 flashes. Therefore high data quality can once again be seen while maintaining high-throughput.

Spectral Scanning Capabilities

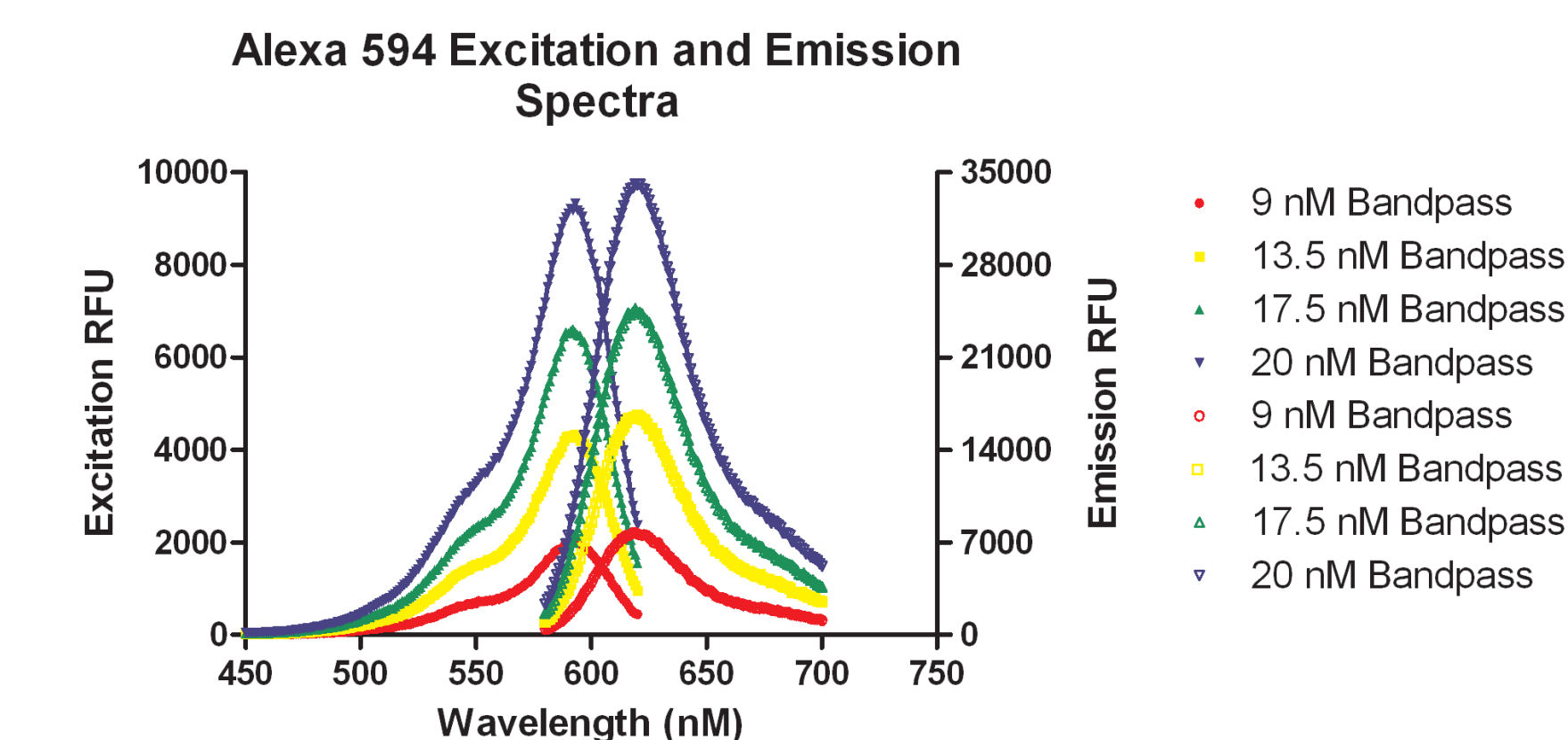


Figure 12 – Spectral scanning experiment

Test – An excitation and emission spectral scan was performed using the Alexa594 Tracer from the Transcreener® ADP² FI Assay. Excitation scans were performed from 450-620 nm in 1nm increments, with emission set at 665 nm. Emission scans were performed from 580-700 nm in 1nm increments, with excitation set at 535 nm. Four separate excitation and emission scans were performed, with excitation and emission monochromator bandpass settings of 9, 13.5, 17.5, and 20 nm.

Summary – By examining the graph of the data, it is evident that excellent spectral scanning results can be achieved with the instrument. Wider bandpass settings can be used for fluors with lower energy, while narrower bandpass settings can be used with fluors possessing narrow Stokes shift.

Conclusions

- The monochromator system of the Synergy™ H4 provides excellent spectral scanning capabilities, as well as the ability to test a variety of wavelength settings with different fluors.
- The filter system provides excellent data quality with the most common fluorescent assay outputs, using read speeds that maintain high-throughput.
- The flexibility and sensitivity of the Synergy™ H4 make the instrument an excellent choice for use in today's life science research laboratory.