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Poster Presentation

The Synergy™HT

A Unique Multi-Detection Microplate Reader for HTS and Drug Discovery

Abstract

Pharmaceutical and biotechnology research requires instrumentation to be both functional and versatile. In the HTS and Drug Discovery environments, microplate-based assays are developed to make determinations on large numbers of samples. Regardless of the assay protocol, the end result is the measurement by some sort of detection device. Towards that end, BioTek has developed Synergy HT Multi-Detection Microplate Reader. Synergy HT utilizes two independent sets of optics to provide uncompromised performance. For absorbance measurements, there is a xenon-flash lamp with a monochromator for wavelength selection and photodiode detection. This allows the selection of any wavelength for endpoint or kinetic measures from 200 nm to 999 nm in 1 nm increments, as well as spectral scans. Traditional visible wavelength fluorescence measurements are made using a tungsten-halogen lamp with interference filters (excitation and emission) for wavelength selection and photomultiplier (PMT) detection. Glow luminescence measurements are also easily accomplished in Synergy HT. If time-resolved or UV excitation fluorescence measurements are required, Synergy HT automatically integrates the xenon-flash-monochromator excitation with the interference emission filter and PMT detection. Typical applications include antibody-antigen binding, receptor-liquid binding, ELISA, nucleic acid quantitation using fluorescent dyes or direct UV analysis. Synergy HT is capable of reading any plate format up to 384-well plates and provides temperature control up to 50° C and shaking as standard features. It is compact (16” W x 15” D x 10” H) and robotics-compatible through the OLE functionality of the KC4™ data reduction software that is bundled with the instrument. An upgrade to CFR 21 Part 11 compliant software is also available.

Figure 1. Synergy™ HT Multi-detection microplate reader

Introduction

The Synergy HT Multi-detection reader is a robotic-compatible microplate reader that can measure absorbance, fluorescence, and luminescence. Its compact design and robot-friendly plate carrier make it an ideal platform for HTS and drug discovery environments (Figure 1). The Synergy HT utilizes a unique dual-optics design (Figure 2). It has both a monochromator/xenon flash system with a silicone diode detector for absorbance, and a tungsten halogen lamp with blocking interference filters and a PMT detector for fluorescence.

The absorbance optics are in a single-channel system that has the capability of measuring absorbance from the UV to the near IR range in all plate formats up to 384-well microplates. The monochromator-based absorbance optics have a wavelength range of 200-999 nm, require no absorbance filters, and can perform spectral scans of substances in increments as small as 1 nm

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(Figure 4). A xenon-flash lamp is used to illuminate a high-precision diffraction-grating monochromator. The monochromatic light is split into an experimental and a reference channel. The experimental channel is next focused onto the microplate well sample, while the reference channel is directed to the reference detector. The unabsorbed light is then focused onto the silicone diode detector (Figure 2). By utilizing the near IR capability of the instrument, sample pathlength can be corrected automatically in liquid samples (Figure 3). This allows for the direct quantitation of unknown sample concentrations from absorbance measurements using extinction coefficients of the analyte rather than a standard curve.

The fluorescence optics are capable of measuring all plate formats up to 384-well plates. The top probe adjusts up and down automatically via software accommodating different plate heights, and different sized probes are available to meet specific applications. A Synergy HT configured for time-resolved fluorescence is capable of measuring fluorescence in either the conventional mode, where the fluorescent emission is measured with the excitation source still present, or in time-resolved mode, where the fluorescence is measured at some point following the cessation of excitation. When the reader is in conventional fluorescence mode, it uses a tungsten-halogen lamp as a light source and band-pass filters in a filter wheel cartridge to provide wavelength specificity. When the reader is used in time-resolved mode, it automatically switches to a xenon-flash lamp light source with a monochromator to select wavelength. The excitation filter cartridge is replaced with the TR-cassette, that, in addition to directing the light from the different light source to the microplate, can also hold a single excitation filter if necessary. When in time-resolved mode, the user has the ability to control the time between the cessation of excitation and the initiation of fluorescence measurement (delay time), as well as the length of time the fluorescent signal is accumulated (collection time). This mode can also be used without a delay to provide excitatory light in the UV range.

Besides special optics, the Synergy HT has numerous features that enhance the reader's capability. Elevated temperatures are regulated by a 4-zone microprocessor-controlled system that ensures superior temperature uniformity up to 50º C. With a compact footprint, a robot-friendly carrier design, and RS-232 connection, the Synergy HT is also compatible with many of the commonly preferred robotic systems. The functionality of Synergy HT is greatly enhanced by its controlling data reduction software, KC4. Using true Object Linking and Embedding (OLE) technology, KC4 can interact directly with Microsoft® Excel and Microsoft® Word programs, providing total control over all report formatting using data objects created by KC4. This allows the user to create custom publication quality reports of microplate applications. In addition to the reporting features, the latest version of KC4 offers real-time monitoring of specific wells during kinetic assays and enhanced well-area scanning. An upgrade to CFR 21 Part 11 compliant software is also available.

Materials and Methods

Reagents and kits were obtained from a variety of sources. Europium chelate standard (cat. # B119-100), samarium chelate standard (cat. # B115-100), and chelate solution (cat. # 1244-105) were purchased from Perkin-Elmer Life Sciences (Boston, MA). Alkaline phosphatase (cat. # P-5521) and potassium dichromate (cat # P-5271) were obtained from Sigma Chemical Company (St. Louis, MO). CSPD®, and Sapphire II™ enhancer were purchased from Applied Biosystems (Foster City, CA). An Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (A-22188) was purchased from Molecular Probes (Eugene, Oregon), while opaque white (cat # 3912), opaque black (cat # 3915), and UV transparent (cat #. 3635) microplates were from Corning-Costar (Cambridge, MA). Sonicated herring sperm DNA (cat. # D1816) was procured from Promega (Madison, WI).

The UV absorbance capabilities of the Synergy HT were demonstrated using nucleic acid measurements. Serial dilutions ranging from 0 to 200 µg/ml of sonicated herring sperm DNA

were made using deionized water as the diluent. After dilution, 200 µl aliquots of each dilution were pipetted in replicates of eight into a Costar 3636 UV-transparent microplate. Using a Synergy HT, the absorbance at 260 nm for each well was determined with and without pathlength correction enabled in the KC4 data reduction software. The resultant raw data, as well as the pathlength corrected data, were plotted using KC4 data reduction software and linear regression analysis was performed.

Measurements of potassium dichromate (K2Cr2O7) demonstrate the spectral scanning feature of the Synergy HT. A stock solution was prepared by dissolving K2Cr2O7 powder in an aqueous 0.05N KOH solution. Several 1:2 dilutions were prepared using 0.05N KOH as the diluent and 200 µl of each dilution aliquoted into wells of a Costar 3635 UV-transparent microplate. Using a Synergy HT, a spectral scan from 200 nm to 600 nm in 1 nm increments was performed using KC4 data reduction software to control reader function and collect the data. These data were then exported to Microsoft® Excel and plotted.

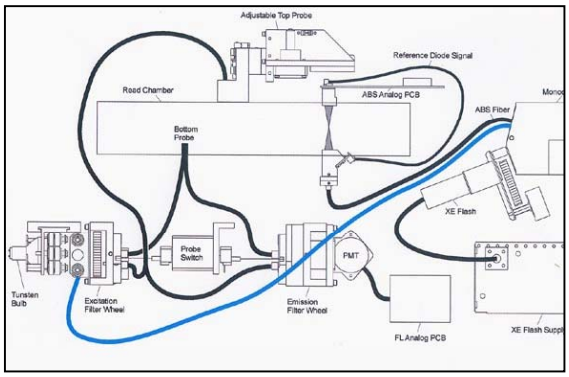

Figure 2. Synergy™ HT Optics

The measurement of hydrogen peroxide using Amplex® Red substrate is an example of the fluorescence capabilities of the Synergy HT. Prior to use, several stock solutions were prepared according to the assay kit instructions. A 10 mM stock solution of Amplex Red was prepared by dissolving Amplex® Red reagent in dimethyl sulfoxide (DMSO). Reaction buffer (1X) comprises of 0.05 M sodium phosphate (pH 7.4) and was prepared from a 5X concentrate supplied by the kit.

Horseradish Peroxidase (HRP) stock solution (10 U/ml) was prepared by dissolving HRP powder in 1X reaction buffer. Note that 1 unit (U) is defined as the amount of enzyme that will form 1.0 mg of purpurogallin from pyrogallol in 20 seconds at pH 6.0 and 20ÞC. Using the supplied ~3% H2O2 solution, a 20 mM working solution was prepared using (1X) reaction buffer as the diluent. A series of dilutions of hydrogen peroxide ranging from 0 to 0.16 µM were made using 1X reaction buffer as the diluent. After the dilutions were prepared, 50 µl of each concentration were placed into wells of a black Corning 3915 microplate.

The reaction was initiated by the addition of 50 µl of a working solution that contained 0.1 mM Amplex® Red reagent and 0.2 U/ml HRP in IX reaction buffer. This results in a final concentration of 50 µM Amplex® Red reagent and 0.1 U/ml of HRP, along with the various concentrations of H2O2 in the reaction mix, and a final reaction volume of 100 µl per well. Samples were incubated for 5 minutes, and endpoint fluorescence was measured using a Synergy HT configured with a 530/25-excitation filter and a 590/35-emission filter, and a sensitivity setting of 50.

Time-resolved fluorescence capabilities were demonstrated using lanthanide chelates. Dilutions of the ready-to-use samarium and europium standards were made using chelate solution as the diluent. The fluorescence of eight replicates of 3000 fmole/well of samarium chelate and 300 fmole/well of europium chelate was measured using a Synergy HT configured for time-resolved fluorescence. An excitation wavelength of 360 nm and either a 620/40 or a 645/40-bandpass-emission filter for europium or samarium, respectively, and a PMT gain setting of 225 were used for time-resolved measurements. Using increasing delay times, measurements were taken using a 20-µsec collection time. For each data point, the mean of chelate-only control wells was subtracted. The resultant fluorescent data were normalized to 1000 by dividing each point by the initial reading and multiplying the ratio value by 1000. The normalized data were plotted using

Microsoft® Excel with a polynomial trend line generated. The effect of increasing collection time was assessed. Using a Synergy HT, the fluorescence of the same microplate with a series of europium dilutions was measured repeatedly with a delay time of 20 µsec and increasing collection times ranging from 20 µsec to 400 µsec. The reader was controlled and the data collected using KC4 data reduction software. After collection, the data were exported to Microsoft® Excel and plotted.

Glow luminescent alkaline phosphatase assays were performed as follows. A series of dilutions ranging from 0.0 to 0.1 U/ml of calf intestinal alkaline phosphatase were made using DEA assay buffer as the diluent. DEA assay buffer consisted of 1 mM MgCl2 and 100 mM DEA pH 9.0 in deionized water. After dilution, 10 ml aliquots of samples and standards were pipetted into the wells of a white Costar 3912 microplate in replicates of eight. Substrate-enhancer mix was prepared fresh by diluting concentrated Sapphire II™ enhancer 1:10 with assay buffer then adding 17 µl of concentrated CSPD® substrate solution to each 1.0 ml of diluted enhancer mix resulting in a final substrate concentration of 0.4 mM. The reactions were then initiated by the addition of 100 µl of enhancer-substrate mixture. The reactions were maintained at 37ÞC and read kinetically from the top every 2 minutes using a Synergy HT with a PMT sensitivity setting of 100. KC4 data reduction software was used to control the reader, collect the data and perform data analysis. Data from the 30-minute read were used as if an endpoint read and plotted using KC4 data reduction software.

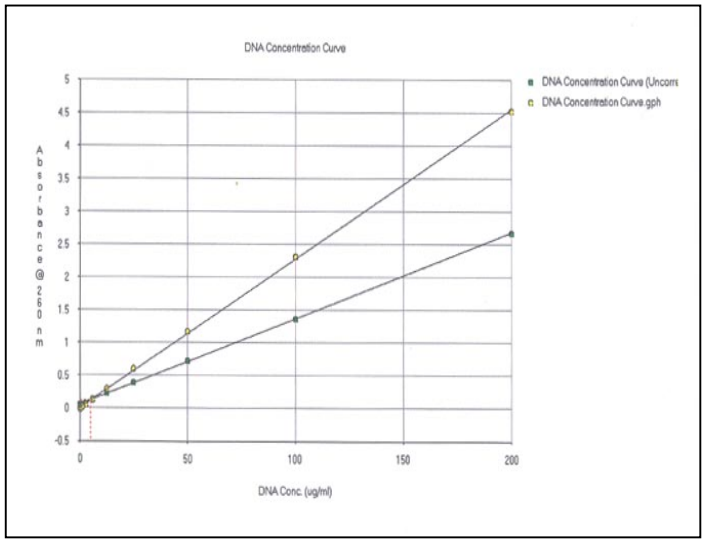

Figure 3. DNA Concentration Curve. Dilutions of herring sperm DNA were prepared using distilled water as the diluent. After dilution, 200 µl aliquots were pipetted into the wells of a Costar 3636, UV-transparent microplate in replicates of eight, and the absorbance at 260 nm determined. Using KC4, absorbance pathlength correction was enabled to correct absorbance values to 1 cm equivalents. Both the raw absorbance, as well as pathlength corrected data, were plotted using KC4 Data reduction software (BioTek Instruments, Winooski, VT).

Results

The data presented in Figure 3 demonstrate the ability of the Synergy HT to measure the UV-absorbance of nucleic acid samples. The raw uncorrected data are linear from 0 to 200 µg/ml. Linear regression analysis reveals a correlation coefficient (r2) of [0.9999] (data not shown).

Detection limits are approximately 0.781 µg/ml, which translates to 156 ng/well. The ability to automatically correct for pathlength allows investigators to correct for pipetting errors while converting directly from absorbance to concentration [1,2]. The accuracy of pathlength correction and direct conversion to concentrations with the use of extinction coefficients is detailed in Table 1. The

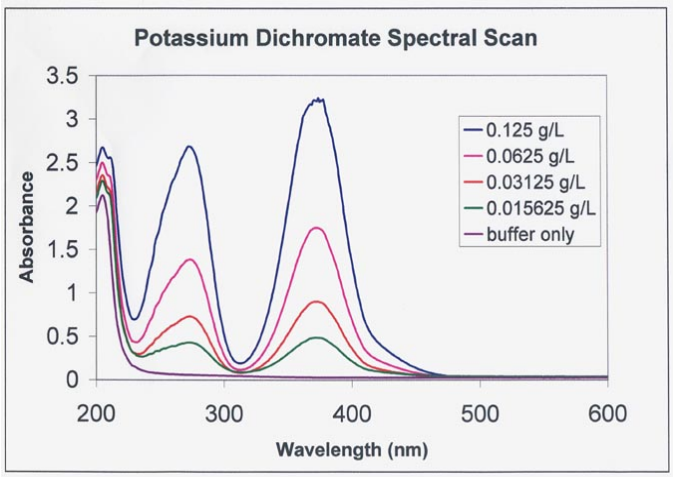

Figure 4. Spectral Scans of Potassium Dichromate Solutions. Spectral scans from 200 nm to 600 nm in 1 nm increments were performed on several dilutions of potassium dichromate dissolved in an aqueous solution of 0.05N KOH. Data from each spectral scan were exported to Microsoft® Excel and plotted.

discrepancy between the DNA concentration values determined in a microplate and corrected for pathlength and a fixed 1-cm pathlength was found to be less than 1%. Because the Synergy HT utilizes a diffraction-grating monochromator, it has the capability of performing absorbance spectral scans on samples in a microplate.

Figure 4 demonstrates the absorbance spectra of several concentrations of potassium dichromate (K2Cr2O7) in an alkaline solution. Two peaks in absorbance can be observed at 273 nm and 377 nm when samples are scanned between 200 nm and 600 nm. The values at these wavelengths are proportional to the K2Cr2O7 concentration. Note that the peak observed at 205 nm is the result of background microplate absorption rather than absorbance of the experimental sample.

As demonstrated in Figure 5, the Synergy HT is capable of fluorescence determinations of samples using standard interference excitation and emission filters and a tungsten halogen light source. The determination of hydrogen peroxide (H_2O_2) concentrations using Amplex Red reagent is an example of the fluorescence capabilities of the Synergy HT. Horseradish peroxidase (HRP) converts hydrogen peroxide to water using an electron donor as a cofactor in a 1:1 stiochiometry. When Amplex red is used as the cofactor, it is converted to the fluorescent compound resorufin and the resultant fluorescence is proportional to the amount of H_2O_2 converted [3]. Using a linear regression, concentrations from 0 to 0.08 µM concentrations can be described with a high degree of confidence, as the correlation coefficient (r2) was calculated to be 0.993. The detection limits for H_2O_2 when using fluorescence were calculated to be 0.004 µM. Taking into account the reaction volume of 100 µl, this concentration can be converted to a detection limit of 400 fmoles/well.

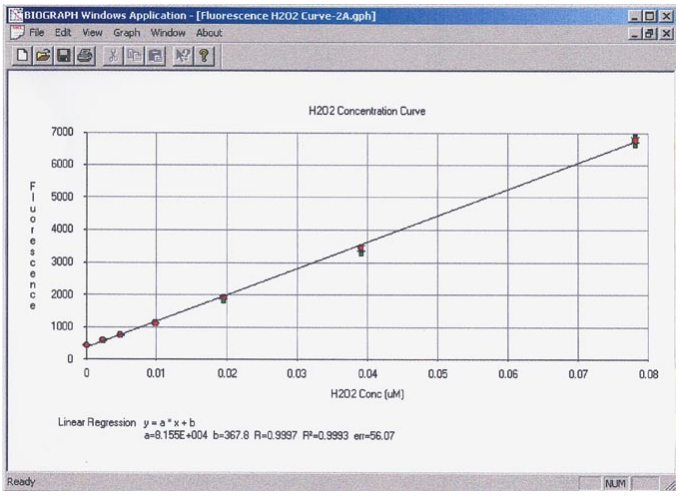

Figure 5. Hydrogen Peroxide Fluorescence Calibration Curve. Hydrogen Peroxide samples (in duplicate) ranging from 0 to 0.08 µM were reacted with HRP and Amplex Red reagent and the fluorescence measured with a Synergy HT using a 530/25-excitation and a 590/35-emission filter. The subsequent data were plotted and linear regression analysis performed using KC4 Data Reduction Software.

The Synergy HT can also perform time-resolved fluorescence measurements when configured with a TR-cassette. In this mode, the delay time and the collection time can be set using KC4 software. The delay time is the time interval from the cessation of the excitation light to the initiation of the read measurement. By keeping the collection time and sensitivity settings constant but changing the delay time, one can observe the decay of fluorescence of compounds with long half-lives, such as the lanthanides (Figure 6). Lanthanide chelates lose their fluorescence as the time from the cessation of excitation to the measurement increases in a first-order kinetic rate. The signal from samarium, which has a shorter lifetime than europium [4], decays much more quickly; when the two lanthanide compounds, each with different t values, are compared, remarkably different decay curves are observed (Figure 6). Another parameter that is available when using the Synergy HT for time-resolved fluorescence measurements is data collection time. This parameter, which is the time that the reader is collecting fluorescent signal, can be as short as 20 microseconds to as long as 16 milliseconds. Using a series of dilutions of europium chelate, the effect of increasing the collection time on the fluorescent signal returned was investigated (Figure 7).

When measuring the fluorescence of europium after a 20-µsec delay, an increase in the fluorescence signal is observed as one increases the collection time. Note that the increase in signal is not necessarily linear relative to the collection time, as the compound's fluorescence is also decaying over time [5]. However, increasing the collection time is a useful means to increase sensitivity when measuring small amounts of lanthanides, particularly those with longer fluorescent half-lives.

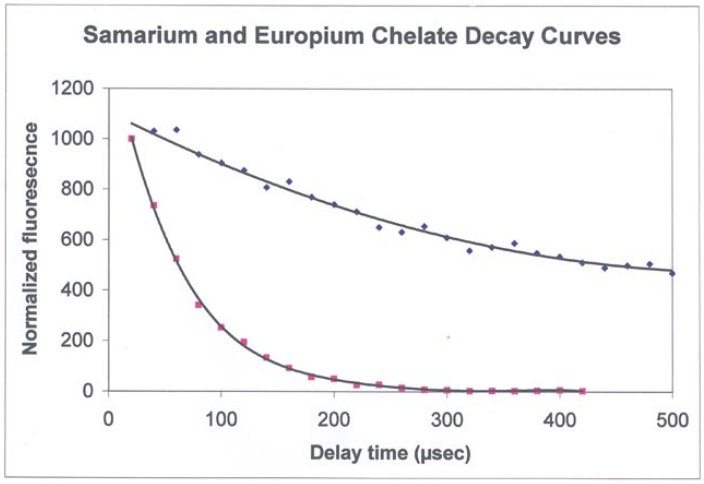

Figure 6. Comparison of Samarium and Europium chelate fluorescence over time. The fluorescence of 3000 fmole/well of samarium chelate (squares) and 300 fmole/well of europium chelate (diamonds) were measured using a Synergy HT with an excitation wavelength of 360 nm and either a 620/40 or a 645/40-bandpass-emission filter for europium or samarium, respectively, and a PMT gain setting of 225. Using increasing delay times, as indicated by the x-axis, measurements were taken using a 20-µsec collection time. For each data point, the mean of chelate-only control wells was subtracted. The resultant fluorescent data were normalized to 1000 by dividing each point by the initial reading and multiplying the ratio value by 1000. The normalized data were plotted using Microsoft® Excel with a polynomial trend line generated. Note that each data point represents the mean of eight determinations.

Figure 8 demonstrates the ability of the Synergy HT to provide accurate and sensitive fluorescence results with UV excitation. The aromatic amino acid tryptophan has an excitation wavelength around 280 nm and a peak emission wavelength of 340 nm. While tungsten-halogen lamps provide very little energy in the UV region, the xenon-flash lamp is quite capable of exciting tryptophan. When the fluorescence of several dilutions of the amino acid is measured, a linear response is observed (Figure 8). Similar results have been obtained measuring tyrosine with an excitation of 276 nm and a 310/20-emission filter (data not shown).

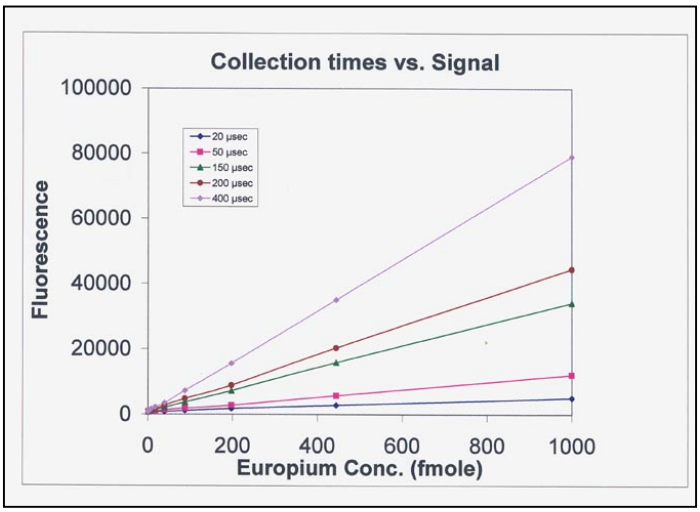

Figure 7. Effect of Collection time on the fluorescence signal of Europium Chelate. Using a Synergy HT, the fluorescence of the same microplate with a series of europium dilutions was measured repeatedly with a delay time of 20 µsec and increasing collections times ranging from 20 µsec to 400 µsec. The reader was controlled and the data collected using KC4 data reduction software. After collection, the data were exported to Microsoft® Excel and plotted.

The Synergy HT can be used to measure glow luminescence reactions. As demonstrated in Figure 9, the enzyme alkaline phosphatase can be detected by using a luminescent substrate such as the 1,2-dioxetane, CSPD. CSPD is dephosphorylated by alkaline phosphatase to an unstable intermediate, which subsequently decomposes slowly and at a fixed rate, which allows for steady-state chemiluminescence that is dependent on the concentration of alkaline phosphatase. Endpoint determinations of alkaline phosphatase, after a 30-minute incubation at 37° C, reveal a linear relationship between enzyme

concentration and luminescence. Using the Synergy HT to control temperature and perform kinetic analysis is demonstrated in Figure 10, which depicts a series of kinetic luminescence determinations of alkaline phosphatase activity. Note that data from one of the wells have been enlarged to provide greater detail.

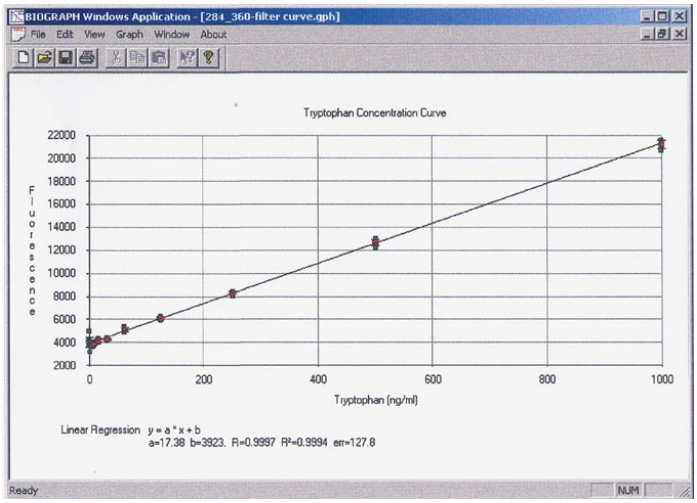

Figure 8. Tryptophan Concentration Curve. Dilution of tryptophan ranging from 0 to 1000 ng/ml were aliquoted into the microplate and the fluorescence measured. Using the UV excitation capabilities of the xenon-flash lamp, tryptophan in solution can be detected using an excitation wavelength of 284 nm and a 360/40 emission filter.

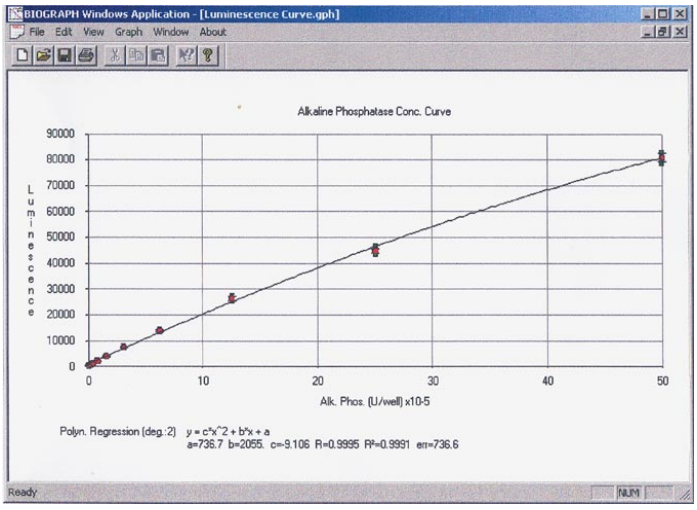

Figure 9. Alkaline Phosphatase Concentration Curve. Dilutions of alkaline phosphatase ranging from 0 to 50 x 10-5 DEA units per well were aliquoted into microplates and CSPD/Sapphire II chemiluminescence reagent added. The luminescence of each sample was determined after a 30-minute incubation.

Discussion

These data presented demonstrate that the Synergy HT Multi-detection reader is a versatile and cost-effective instrument for today's research environment. It has the capability to perform many of the most utilized spectroscopic methods, including absorbance, fluorescence, time-resolved fluorescence, and glow luminescence. With two complete sets of optics, there are no compromises in terms of performance when switching from absorbance to fluorescence. Integration of the two optics allows the reader to be used for time- resolved fluorescence, as well as UV-excitation fluorescence, providing greater functionality. While we have demonstrated the ability to measure the aromatic amino acid tryptophan in solution, this methodology can be applied to peptides and proteins. Tryptophan, and to a lesser extent tyrosine, are responsible for protein fluorescence [5]. The environment in which tryptophan exists in a protein structure can influence the peak excitation wavelength. Having the ability to alter the excitatory wavelength of the monochromator in 1 nm increments allows the investigator to optimize the signal.

Absorbance measurements are performed using dedicated optics that include a monochromator. This allows for measurements to be made at peak wavelengths rather than at the nearest available filter wavelength. The wavelength bandpass of the monochromator is 2 nm, which is considerably narrower than the 10 nm that traditional bandpass filters provide. BioTek's unique enhanced reading mode provides accuracy and precision for wells with high absorbance values. This, along with the dual beam reference-optics, provides accurate and consistent results.

The reader can read any plate format up to 384-wells, automatically adjusting the top probe height setting to accommodate different plates when reading fluorescence from the top. Temperature control up to 50º C using a 4-zone microprocessor-controlled system results in well-to-well temperature uniformity. Programmable shaking is also a standard feature for the Synergy HT. The Synergy HT has been designed from the ground up for robotics compatibility, with a fully extending plate carrier and interfacing via the OLE capabilities of the KC4 software used to control the reader. For those labs that require CFR 21 Part 11 compliance, a version of KC4 is also available.

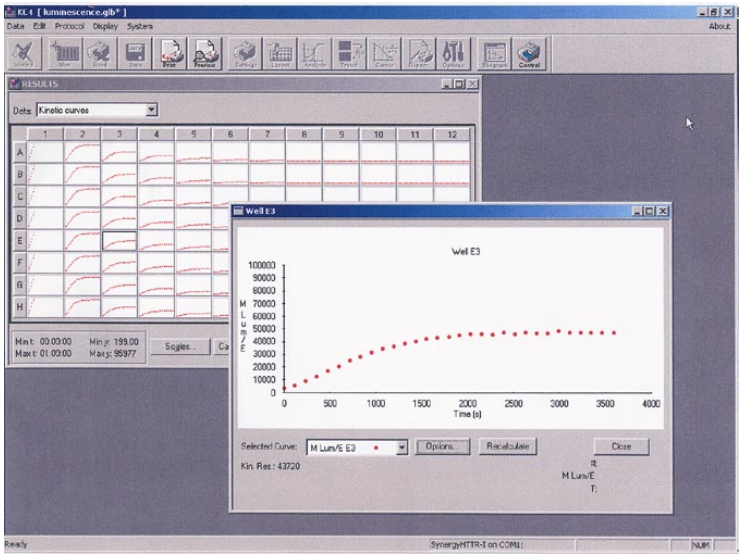

Figure 10. Change in luminescence over time. Luminescence determinations of alkaline phosphatase reactions ranging from 0 to 1 x10-3 DEA units/well) were made kinetically every 2 minutes for 60 minutes. Note that one kinetic well has been enlarged. Reactions were prepared as described in materials and methods.

Table 1.

Sample (µg/ml)	Raw OD	Corrected OD	DNA Conc. (µg/ml)	Expected Conc.
1	1.347	2.308	115.4	
2	1.369	2.344	117.2	
3	1.376	2.336	116.8	
4	1.368	2.300	115.0	116.4
5	1.368	2.321	116.0	
6	1.383	2.302	115.1	
7	1.378	2.293	114.7	
8	1.410	2.303	115.1	
		Average	115.7	

Table 1. Comparison of DNA concentrations determined by automatic pathlength correction to fixed 1 cm pathlength. The absorbance at 260 nm of a DNA sample (200 µl/well) was measured using a Synergy HT with pathlength correction enabled in replicates of eight. An aliquot of the same sample was placed in a Bio-Cell, which provides a 1 cm pathlength and the absorbance measured. The resultant corrected data for each vessel were then directly converted to concentration.

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(3) Zhou, M., Diwu, Z., Panchuk-Voloshina, N., and Haugland, R.P. (1997) A Stable Nonfluorescent Derivative of Resorufin for the Fluorometric Determination of Trace Hydrogen Peroxide: Applications in detecting the Activity of Phagocyte NADPH Oxidase and other Oxidases, Analytical Biochemistry, 253:162-168.

Application Note: Stability of the Wallac LANCE™ Eu-chelates, Perkin-Elmer Life Sciences, http://www.lifesciences.perkinelmer.com/library/appnotes.asp

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