BioTek
Get a Better Reaction.

Poster Presentation

The Synergy™ 2, A Novel Approach to Microplate Multi-Detection for HTS and Drug Discovery

Paul Held and Xavier Amouretti, BioTek Instruments, Inc., Winooski, VT 05404

Abstract

The Synergy™ 2 is a new type of reader that provides research laboratories performances usually found on high-end HTS instrumentation, while delivering flexibility and efficient cost-control to screening laboratories. The Synergy 2 utilizes multiple optics to provide performance regardless of the detection technology. Absorbance measurements use a xenon lamp with a monochromator for wavelength selection from 200 nm to 999 nm. Fluorescence measurements are made using either a tungsten-halogen or a xenon-flash lamp with filters and dichroic mirrors and a PMT for detection. Fluorescence polarization is accomplished with polarizing filters in conjunction with dichroic mirrors. For time-resolved fluorescence measurements, the Synergy 2 integrates a high-energy xenon-flash lamp with excitation and emission filters and PMT detector. Luminescence measurements are accomplished by using a liquid-filled light guide. The Synergy 2 is capable of reading any plate format up to 1536 wells, is robotic compatible and provides temperature control and shaking. Reagent injectors are also available.

Figure 1. Synergy™ 2 Multi-Detection Microplate Reader.

 TEL: 888-451-5171 FAX: 802-655-7941 Outside the USA: 802-655-4740 E-mail: customercare@biotek.com

Introduction

Multi-mode microplate readers are designed either for screening or for research applications. In the first instance, they are fast end-point readers that use homogeneous detection technologies. Readers designed for research applications are more flexible but with lower levels of performance. The Synergy 2 is a new type of reader that provides the combined benefit of bringing to research laboratories performances and technologies usually found on high-end HTS instrumentation, while at the same time delivering flexibility and efficient cost-control to screening laboratories. The Synergy 2 utilizes multiple sets of optics to provide optimal performance regardless of the detection technology. Absorbance measurements use a xenon-flash lamp with a monochromator for wavelength selection, allowing the selection of any wavelength for endpoint or kinetic measures from 200 nm to 999 nm. Fluorescence measurements are made using either a tungsten-halogen lamp or a xenon-flash lamp with bandpass filters with or without dichroic mirrors for wavelength selection and PMT for detection. As little as 1 pM fluorescein can reliably be detected. Fluorescence polarization is accomplished with the use of polarizing filters in conjunction with label specific dichroic mirrors for wavelength specificity. For time-resolved fluorescence measurements, the Synergy 2 integrates a high-energy xenon-flash lamp with excitation and emission filters and PMT detector. Concentrations of europium as low as 60 fM can be detected. Luminescence measurements are made using a liquid-filled optical fiber to capture light along with a low noise PMT. Detection limits of 30 amol of ATP have been observed. The Synergy 2 is capable of reading plate formats up to 1536 wells, is robotic compatible, provides temperature control, and includes shaking as standard features. Examples of typical performance of the instrument, including fluorescence polarization; DLR luminescence; LANCE, and DELFIA time-resolved fluorescence assays will be provided, along with an overview of the system.

We have used the analyte adenosine 3', 5' cyclic monophosphate (cAMP) to demonstrate the ability of the Synergy 2 to perform several different types of reading measurements. Adenosine 3', 5' cyclic monophosphate (cAMP) plays a critical role in the transmission of signals by functioning as a "second messenger". Binding of hormones or ligands to their respective receptors can either inhibit or enhance the production of cAMP by changing the activity of the enzyme adenylate cyclase. Adenylate cyclase is a membrane-associated enzyme that catalyzes the formation of cAMP from ATP. Intracellular cAMP levels, in turn, regulate enzymatic activity of numerous protein kinases, which phosphorylate specific targets setting off a cascade of cellular events. Because of the importance of cAMP as a cellular messenger, several different reading methodologies have been developed to measure cellular levels. Here we describe some of these methodologies and demonstrate the ability of the Synergy 2 to quantitate cAMP using these methodologies.

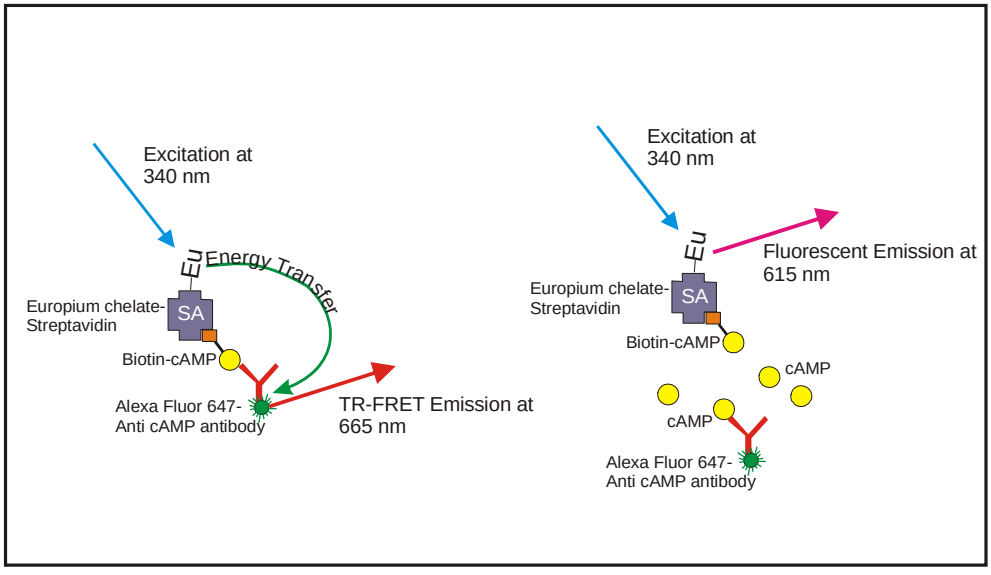


Figure 2. LANCE Assay Diagram. Pulsed light at 340 nm excites the europium chelate of the europium-streptavidin/Biotin-cAMP tracer. The energy emitted from the Eu-chelate is transferred to the Alexa-Fluor® 647 labeled anti-cAMP antibody bound to the tracer, generating a TR-FRET signal at 665 nm. Residual energy from the Eu-chelate will produce light at 615 nm. In the presence of cAMP, the cAMP will compete with the tracer for the antibody binding sites.

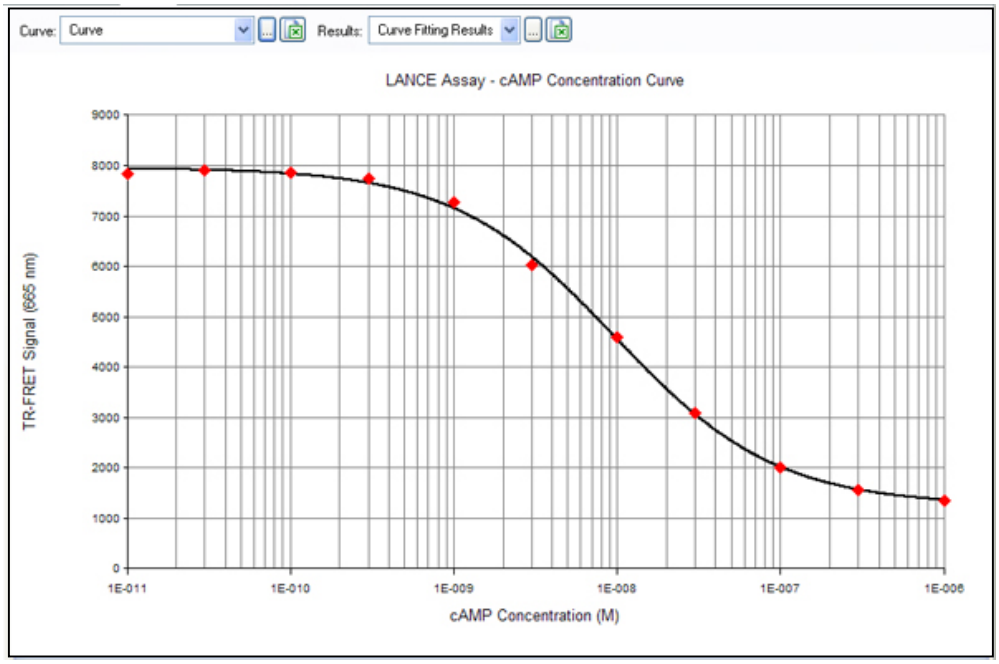


Figure 3. Concentration Curve of cAMP. The TR fluorescence was measured using a Synergy™ 2 Multi-Detection Microplate Reader. The samples were excited using a xenon flash lamp with a 320/40 nm excitation filter. The emission data was collected for 200 μsec after a delay time of 50 μsec using a 665/10 nm emission filter. The TR-FRET signal at 665 nm was plotted against cAMP concentration using Gen5™ Data Analysis Software.

The LANCE cAMP assay is a homogenous Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) immunoassay designed to measure cAMP. The assay is based on the competition between a europium-labeled cAMP tracer complex and sample cAMP for binding sites on cAMP-specific antibodies labeled with the dye Alexa Fluor® 647 (Figure 2). The europium-labeled cAMP tracer complex is formed by the interaction between Biotin-cAMP (B-cAMP) and streptavidin labeled with europium chelate (Eu-SA). When antibodies are bound to the Eu-SA/B-cAMP tracer, 340 nm light excites the Eu-chelate molecules of the tracer. The energy is transferred to an Alexa molecule on the antibodies, which in turn emits light at 665 nm. The fluorescence intensity measured at 665 nm will decrease in the presence of cAMP from test samples. Thus the fluorescence intensity at 665 nm will be inversely proportional to the cAMP concentration of the sample.

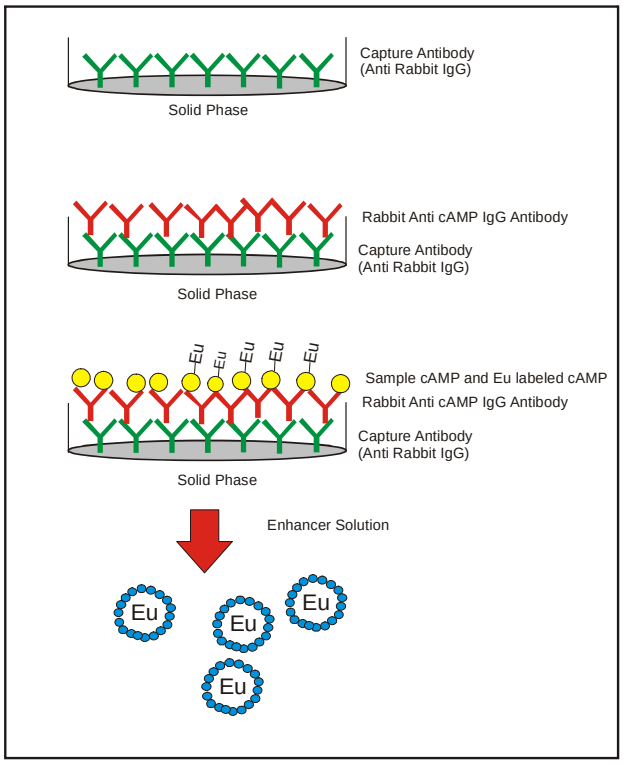


Figure 4. The DELFIA® Assay Diagram. The DELFIA® cAMP assay is based on the competition between europium-labeled cAMP and sample cAMP for a limited number of binding sites on cAMP specific polyclonal antibodies derived from rabbits. A second antibody, directed against rabbit IgG, is used to coat the solid phase and capture the rabbit antibody complex, allowing separation of the antibody-bound and free antigen by washing with a microplate washer. With the addition of enhancement solution, europium ions disassociate from the labeled antigen into solution, forming fluorescent chelates with components in the enhancement solution.

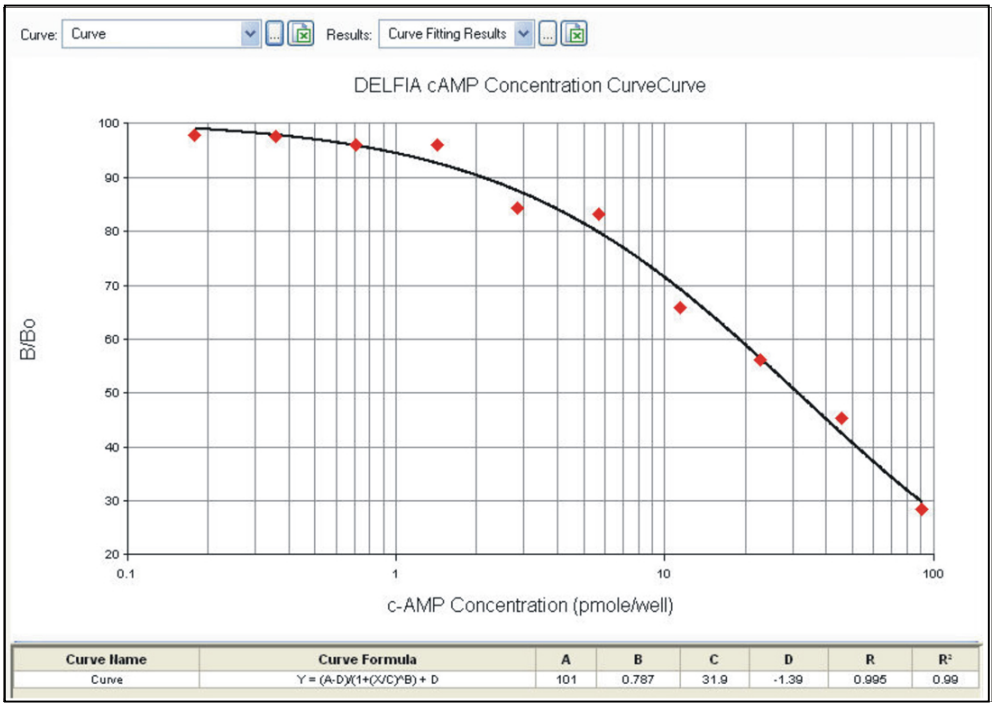


Figure 5. DELFIA cAMP Concentration Curve. The TR-fluorescence was measured using a Synergy™ 2 Multi-Detection Microplate Reader. The samples were excited using a xenon flash lamp with a 360/40 nm excitation filter. The emission data was collected for 1000 μsec after a delay time of 250 μsec using a 620/40 nm emission filter.

The DELFIA® cAMP assay is a solid phase, time resolved fluoroimmunoassay based on the competition between europium-labeled cAMP and sample cAMP for a limited number of binding sites on cAMP specific polyclonal antibodies derived from rabbits (Figure 4). A second antibody, directed against rabbit IgG, is used to coat the solid phase and to capture the rabbit antibody complex, allowing separation of the antibody-bound and free antigen. With the addition of enhancement solution, europium ions disassociate from the labeled antigen into solution, forming fluorescent chelates with components in the enhancement solution. When the fluorescence is measured, there is an inverse relationship between fluorescent signal and cAMP concentration.

The fluorescent polarization cAMP assay is a homogeneous fluorescent assay designed to quantitate cAMP in solution. The assay is based on the competition between fluorescein-labeled cAMP and non-labeled sample cAMP for a fixed number of antibody binding sites (Figure 6). Fluorescence polarization (FP) is a fluorescent detection technique that makes measurement through polarizing filters that are parallel and perpendicular to the plane of a polarized excitation source. Polarization values for any fluorophore complex are inversely related to the speed of molecular rotation of that complex. Because the speed of rotation is also inversely related to the size of the molecule, polarization values will be high with large molecule complexes and low with small molecules.

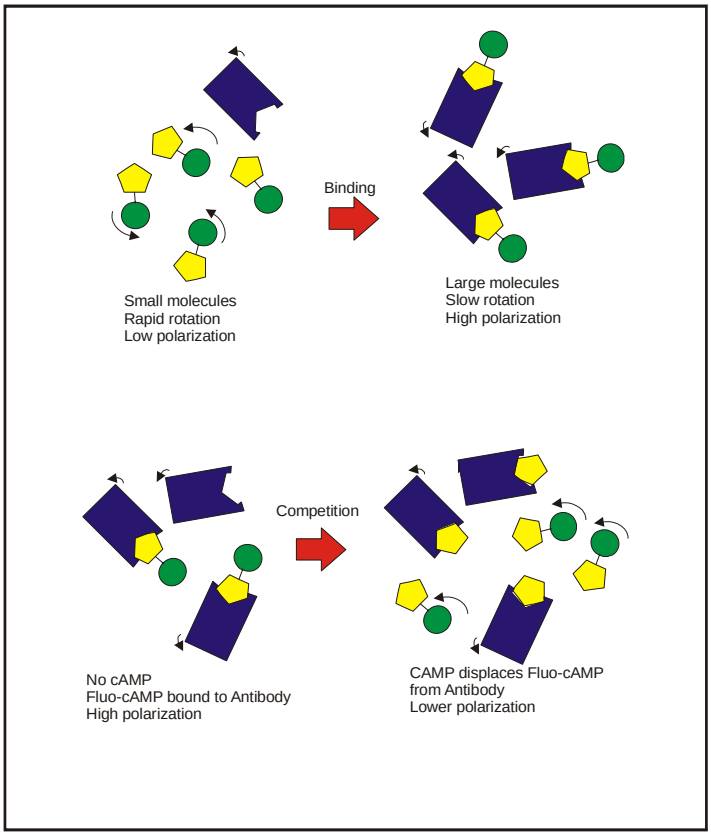
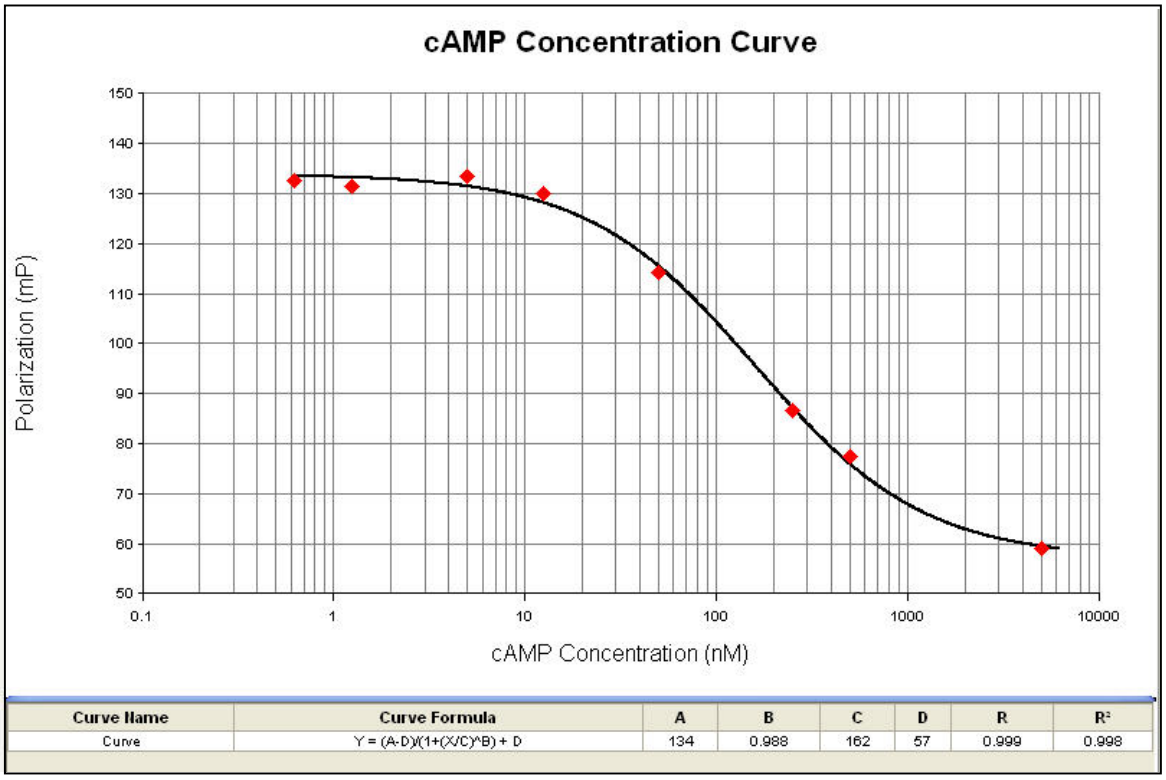


Figure 6. The Quantitation of cAMP Using Fluorescence Polarization. Because the speed of rotation is inversely related to the size of the molecule, polarization values will be high with large molecule complexes and low with small molecules. The cAMP assay is based on the competition between fluorescein-labeled cAMP (8-fluo-cAMP) and non-labeled sample cAMP for a fixed number of antibody binding sites. As increasing amounts of unlabeled cAMP displace the 8-fluo-cAMP polarization values decrease.



Curve Name	Curve Formula	A	B	C	D	R	R^2
Curve	Y = (A-D)/(1+(X/C)^B) + D	134	0.988	162	57	0.999	0.998

Figure 7. Typical Standard Curve of cAMP Using Fluorescence Polarization. Polarization was calculated after measuring the fluorescence through parallel and perpendicular polarizing filters. Polarization was then plotted as a function of cAMP concentration.

Materials and Methods

Several different cyclic adenosine monophosphate (cAMP) assay kits were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). These include a $[FP]^2$ ™ Fluorescent polarization cAMP assay kit (catalog Number FPA202), a LANCE™ cAMP 384 kit (catalog number AD0262), and a DELFIA® cAMP kit (catalog number CR89-102). DLR and Dual Glo luciferase kits were procured from Promega (Madison, WI), while NAD and NADH were obtained from Sigma-Aldrich. Dual-Luciferase Reporter Assay System (P/N E1910) was purchased from Promega Corporation (Madison WI). Purified recombinant firefly enzyme (Quantilum) was procured from Promega, while recombinant Renilla luciferase (Novalite®) was from Chemicon (Temecula, CA). All experiments used Corning Costar 3912 white opaque microplates. Unless provided specifically by the assay kit, all microplates were purchased from Corning (Corning, NY).

The DELFIA assay for cAMP was performed according to the kit instructions. Briefly, 50 μl of Anti-cAMP serum solution was added to each well, except to the blank and non-specific binding (NSB) wells of the Anti-rabbit IgG microplate supplied with the kit. A total of 200 μl of assay buffer was added to the blank wells, while 100 μl was added to the NSB wells. The 96-well plate was placed in the Synergy 2 reader and set to shake at a setting of 1 for 30 minutes while the cAMP standards were diluted. A series of dilutions of cAMP ranging from 0 to 91 pmoles/50 μl were made from the stock cAMP solution provided by the kit using cAMP buffer. In replicates of 8, 50 μl aliquots of each dilution were added to the plate, as well as 100 μl of a Eu-tracer solution provided by the kit. The plate was allowed to incubate with shaking for 60 minutes at room temperature. After incubation, the plate was washed 4 times with 300 μl of wash buffer supplied with the assay kit using an ELx50™ Automated Strip Washer (BioTek Instruments, Winooski, VT). After the final aspiration, 200 μl of enhancement solution was added directly from the supplied reagent bottle. The plate was shaken for 5 minutes, and then read in time resolved mode using a Synergy™ 2 Multi-Detection Microplate Reader.

The LANCE assay for cAMP was performed according to the kit instructions. A series of serial dilutions of cAMP from 0 to 1 x 10^{-6} M was prepared as described by the kit instructions using PBS as the diluent. Aliquots (6 μl) of samples and standards were aliquoted in replicates of 8 into the solid black opaque 384-

well microplate provided by the assay kit. In addition, 6 µl of previously prepared working antibody solution was also added to all of the assay wells and the resultant mixture was allowed to incubate at room temperature for 60 minutes. The working antibody solution was prepared by diluting the Alexa Fluor 647 labeled anti-cAMP antibody 1:100 in PBS. After incubation, 12 µl of detection mix was added to all of the wells of the microplate. The detection mix was prepared prior to use by diluting the Eu-W8044 labeled streptavidin and Biotin-cAMP reagents as outlined in the assay kit instructions. Once the reagents were added to the microplate, the plate was sealed with an adhesive plastic film and allowed to incubate overnight at room temperature. The following morning the plate was measured in time resolved mode with a 340/30 nm excitation filter and a 665 nm emission filter.

The FP assay for cAMP was performed according to the kit instructions. A series of serial dilutions of cAMP from 0 to 500 nM was prepared as described by the kit instructions using PBS as the diluent. Aliquots (10 µl) of samples and standards were aliquoted in replicates of 8 into the solid black polystyrene opaque 384-well microplate provided by the assay kit. In addition, 10 µl of previously prepared stimulation mix without IBMX was also added to all of the assay wells and the resultant mixture was allowed to incubate at room temperature for 30 minutes. After incubation, 20 µl of detection mix was added to all of the wells of the microplate. The detection mix was prepared prior to use by diluting the Fluo-cAMP tracer in detection buffer (supplied by the kit) as outlined in the assay kit instructions. Once the reagents were added to the microplate, the plate was sealed with an adhesive plastic film and allowed to incubate overnight at room temperature. The following morning the plate was measured in fluorescence polarization mode using a 485/20-excitation and a 528/20-emission filter. In addition, a 510 nm dichroic mirror (P/N 7137510) was used to further discriminate the excitation and emission signals. Measurements were made through both a parallel and perpendicular polarizing filters. The degree of polarization was then calculated after blank subtraction.

Purified Firefly and *Renilla* luciferase enzymes were diluted to approximate equal molarity and mixed at various molar ratios. After mixing, 20 µl of each of the mixtures was then aliquoted into wells of a microplate in replicates of 8. Using the injection system of the Synergy 2, 100 µl of Firefly Luciferase substrate LRL was added and the luminescent signal measured for a total of 10 seconds. Following the completion of the read, 100 µl of Stop and Glo reagent was added. This reagent terminates Firefly luciferase signal, as well as provides the substrate necessary for Renilla luciferase. Following a second delay after the Stop and Glo reagent injection, Renilla luciferase signal was measured for a total of 10 seconds. Data for both measurements was then exported to Microsoft Excel for analysis.

Measurements of nicotinamide adenine dinucleotide (NAD^+) nicotinamide adenine dinucleotide (reduced) (NADH) were taken to demonstrate the spectral scanning feature of the Synergy 2. A stock solution was prepared by dissolving NAD^+ or NADH powder in an aqueous solution and 200 µl of each dilution aliquoted into wells of a Costar 3635 UV-transparent microplate. Using a Synergy 2, a spectral scan from 200 nm to 999 nm in 1 nm increments was performed using Gen5™ Data Analysis Software to control reader function and collect the data.

Results

As demonstrated in Figure 3, the Synergy™ 2 Multi-Detection Microplate Reader is capable of performing TR-FRET measurements. When using this mode the xenon flash lamp excites the donor europium molecule. A delay between the cessation of excitation light and the initiation of reading is possible because of the lengthy half-life of the europium fluorescence. The energy is transferred to the acceptor Alexa-fluor only when the antibody is bound to the biotin-cAMP moiety. The energy transfer is measured as a fluorescent signal at 665 nm, whereas the normal emission for europium is at 615 nm. Increasing amounts of cAMP result in a decrease in measured signal, which is consistent with the competitive nature of the assay. Increasing amounts of exogenous cAMP compete with Biotin-cAMP for a limited number antibody binding sites. Only when the Biotin-cAMP is bound to both the fluorescent antibody and the streptavidin-europium complex will TR-FRET take place.

Straightforward Time-Resolved (TR) measurements can also be performed using the Synergy 2. As depicted in Figure 5, DELFIA assays can be reliably measured using the Synergy 2. DELFIA assays use a solid phase antibody interaction to allow for plate washing to remove unbound material. Europium labeled cAMP and exogenous cAMP compete for a limited number of antibody binding sites, resulting in an inverse relationship between the amount of europium retained and cAMP concentration. When the enhancement solution is added after washing, the captured europium is released and forms chelates with

reagents in the enhancement solution. These chelates can then be measured by time resolved fluorescence. The delay between the cessation of excitation and the collection of data allows for background fluorescence to dissipate. As seen in Figure 5, as increasing amounts of cAMP are added to the wells, a decrease in TR- fluorescence is observed. In addition the background fluorescence is quite low.

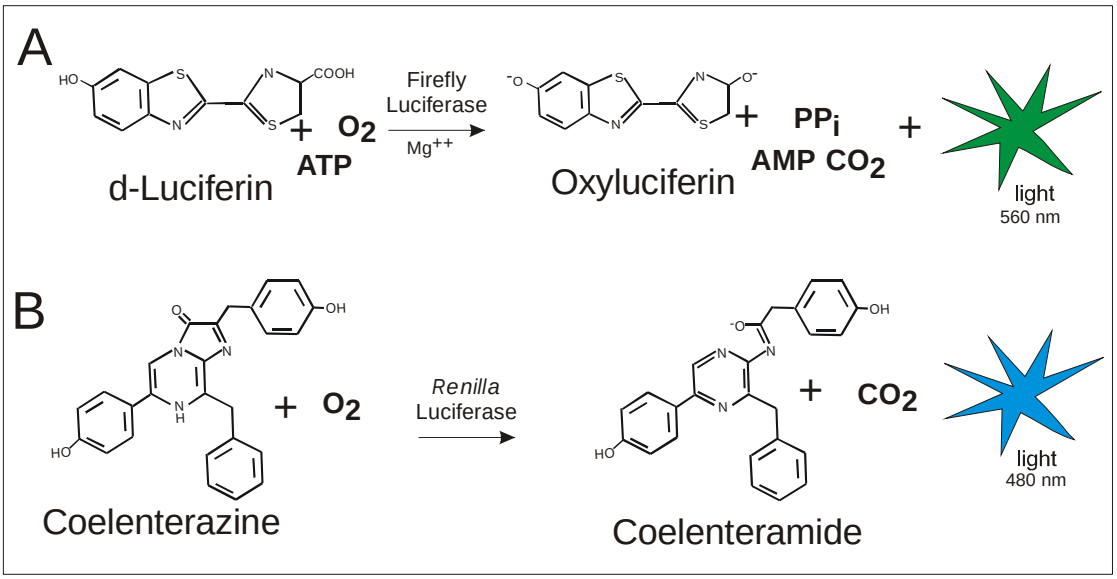


Figure 8. Bioluminescent Reactions Catalyzed by Firefly and *Renilla* Luciferase. (A) Firefly luciferase, using ATP, catalyses the two-step oxidation of luciferin to oxyluciferin, which yields light at 560 nm. (B) *Renilla* luciferase catalyses the oxidation of coelenterazine to coelenteramide, which yields light at 480 nm.

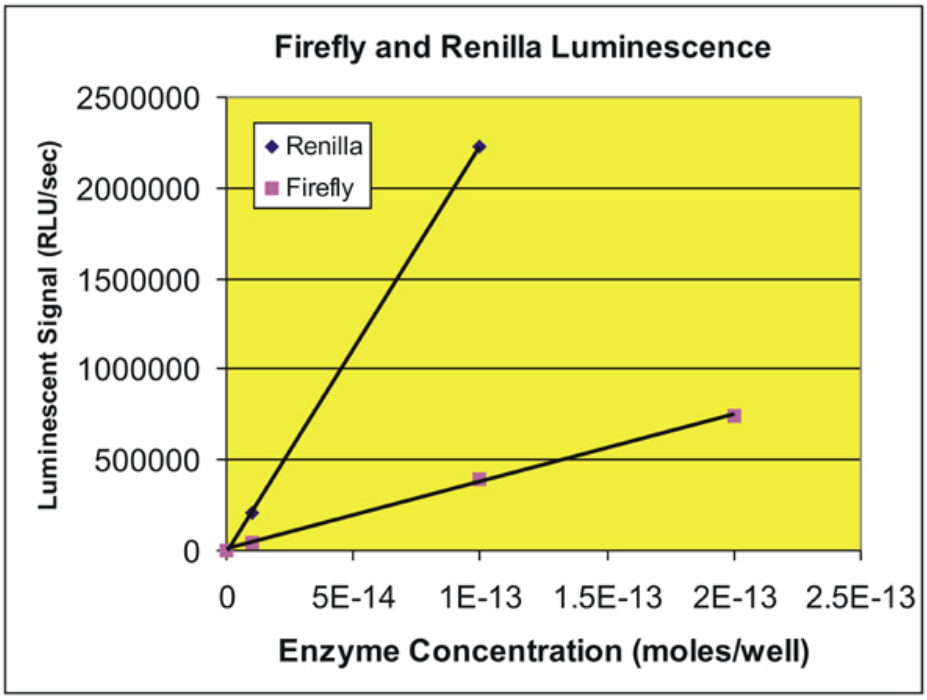


Figure 9. Firefly and Renilla Flash Luminescence. Promega Flash DLR assays were performed on samples with various concentrations of both Firefly and Renilla luciferase enzymes. Luminescence was determined using a Synergy™ 2 Multi-Detection Microplate Reader with dual reagent dispenser module.

Fluorescence Polarization (FP) can also be used to determine cAMP levels. Small fluorescently labeled cAMP moieties have a relatively low polarization value, which increases significantly when it is bound to a large molecule such as an antibody. Exogenous unlabeled cAMP would be expected to compete for these antibody sites. Thus samples with low levels of cAMP would have the fluorescently labeled fluor primarily bound to the antibody, while samples with high levels would have mostly unbound fluor. As demonstrated in Figure 7, there would be an expected decrease in polarization values with increasing amounts of cAMP, as more and more of the small rapidly rotating unbound fluor is available in solution.

The ability of the Synergy 2 to perform both flash and glow type luminescent measurements is demonstrated in Figures 9 and 10 respectively. Flash luminescent measurements require that dedicated reagent injectors add precise amounts of fluid at specific times relative to the measurement of luminescence. The DLR or Dual luciferase assay requires that two different reagents be added with subsequent measurements taking place after each reagent addition. As demonstrated in Figure 9, there is a linear relationship between luminescent output and enzyme concentration for both Firefly and Renilla luciferase. This occurs even though both enzymes are present in the same well. Figure 10 demonstrates the ability of the Synergy 2 to make end-point luminescent determinations on Firefly and Renilla containing samples.

The Synergy 2 reader can also perform absorbance measurements, such as spectral scans. As seen in Figure 11, there is a remarkable difference between the oxidized (NAD+) and reduced (NADH) forms of nicotinamide adenine dinucleotide. When spectral scans from 200 nm to 999 nm are performed using the Synergy 2, NADH demonstrates a very distinct absorbance peak at 340 nm, not observed with NAD+. The small but distinct peak observed at 977 nm is the result of water in the sample.

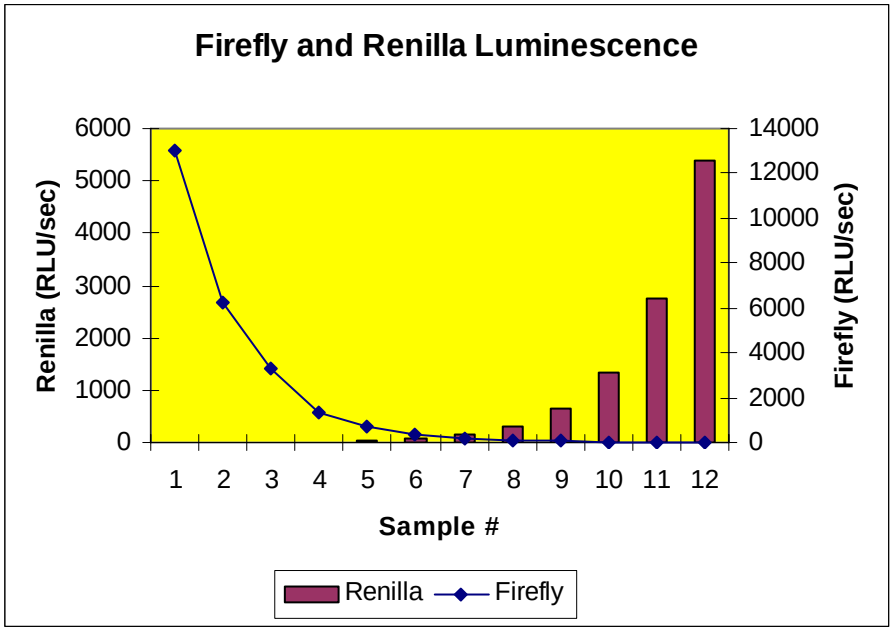


Figure 10. Firefly and Renilla Glow Luminescence. Firefly and Renilla luciferase activity were measured using a Promega Dual Glo assay kit. Each sample had a mixture of Firefly and Renilla enzyme. Reagents were added manually in sequence (Firefly first), with subsequent luminescent determinations after the addition of each luciferase reagent. Data was exported to Microsoft Excel and plotted.

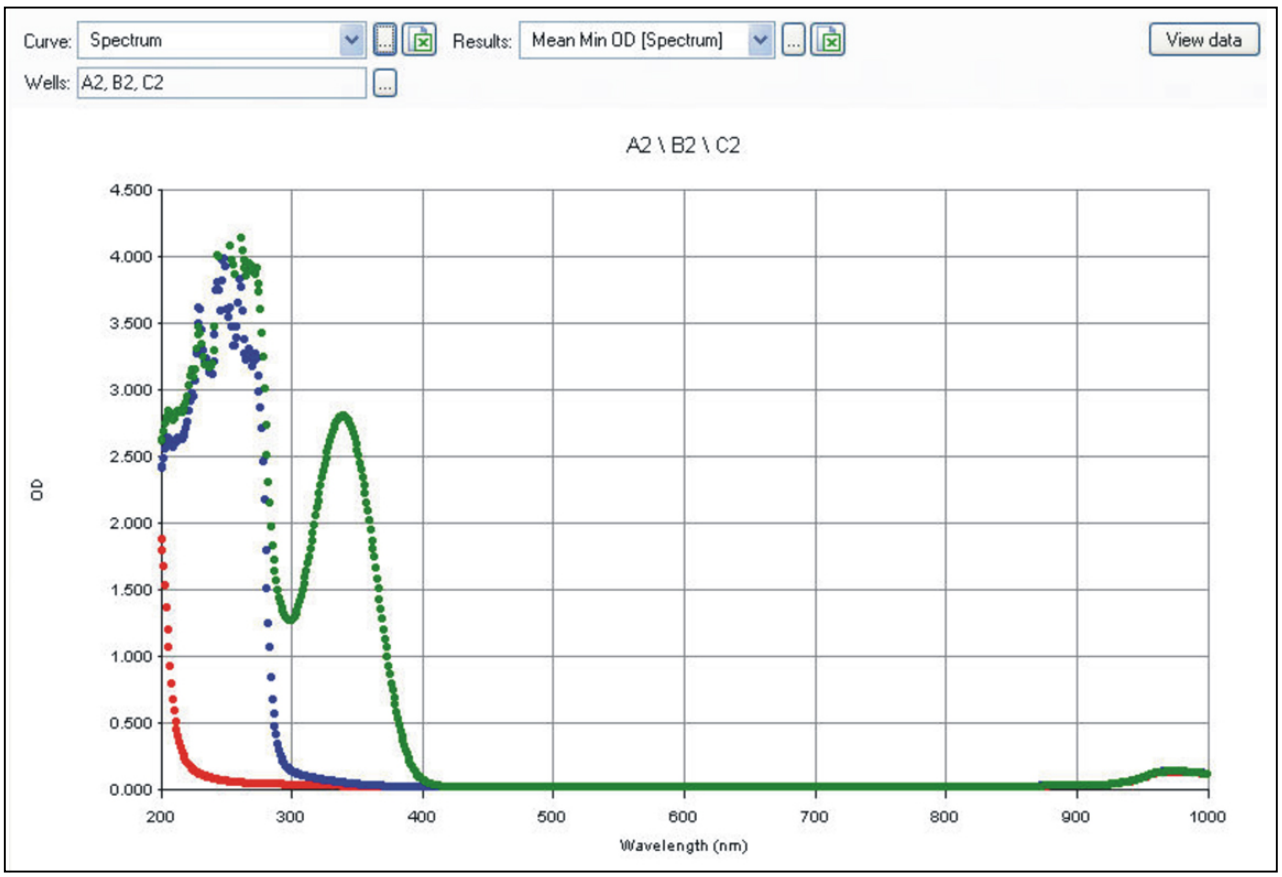


Figure 11. Spectral Analysis of NAD+ and NADH. Equimolar amounts of NAD^+ (blue curve) and NADH (green curve) were dissolved in aqueous solution. Spectral analysis of each sample along with a water only control (red curve) was performed using a Synergy™ 2 Multi-Detection Microplate Reader. The reader was controlled and data analysis performed using Gen5™ Data Analysis Software.

Rev. 7/27/06