Synergy™ HT, A Microplate Multi-Detection Reader for Today's High Performance Luminescence Research

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Introduction

Luminescent assays are sensitive and quantitative tools commonly used for a variety of purposes in biomedical and pharmaceutical research. Quantitation of ATP can be used to detect and measure cellular growth, as well as bacterial contamination. Luminescent genetic reporting assays are widely used to study gene expression and cellular responses to external stimuli in Prokaryotic and Eukaryotic organisms. Dual-reporter assays use two independent reporter systems simultaneously to improve experimental accuracy. One reporter is used to measure the response resulting from the experimental conditions and is often referred to as the "experimental" reporter. The second reporter is designed not to respond to the experimental conditions, acting as an internal control from which data generated by the experimental reporter can be normalized. Normalization of the data serves to compensate for variability caused by differences in transfection efficiency, cell viability, cell lysis, and pipetting. Promega's Dual-Luciferase® System uses the activities of luminescent proteins (luciferases) from the firefly (*Photinus pyralis*) beetle and the sea pansy (*Renilla renformis*) to serve as an experimental and a control reporter respectively (See Figure 1).

The Synergy™ HT Multi-Detection Reader (Bio-Tek Instruments, Winooski, VT) is a robotic compatible microplate reader that can measure absorbance, fluorescence, and luminescence in all plate formats up to 384-well plates with exceptional performance in all three detection modes, that has recently been optimized for luminescence measurements (Figure 2). The Synergy HT utilizes a unique dual optics design that 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 photomultiplier tube (PMT) detector for fluorescence. Reagent addition is accomplished by an optional external injector module, which controls two independent injector syringes. Each syringe is connected to a separate injector tip. Emitted luminescence is captured using either the top or bottom probes and measured using the Synergy HT's low noise PMT operated in photon integration mode. Here we describe the use of a Synergy™ HT Multi-Detection Microplate Reader (Bio-Tek Instruments) to perform dual-luciferase measurements with purified recombinant enzymes.

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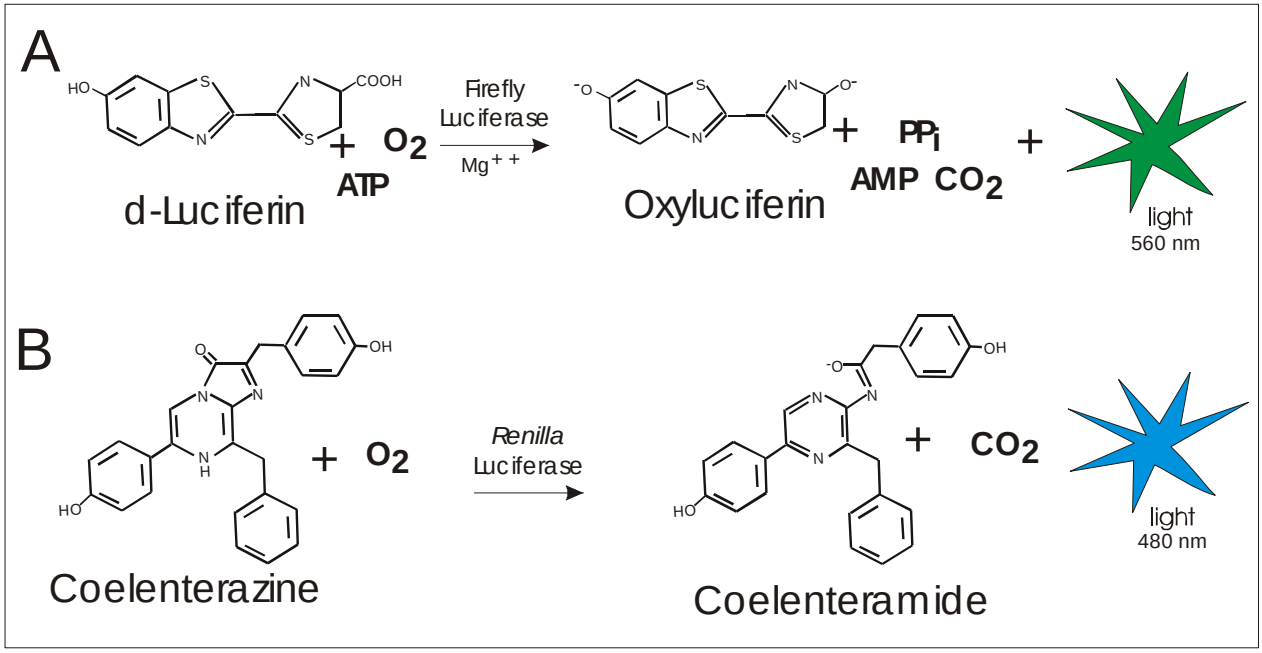


Figure 1. Bioluminescent Reactions Catalyzed by Firefly and *Renilla* Luciferase. (A) Firefly luciferase, using ATP, catalyses the two-step oxidation of luciferin to oxyluciferin, which yields light at 560 nm. (B) *Renilla* luciferase catalyses the oxidation of coelenterazine to coelenteramide, which yields light at 480 nm.

Figure 2. Synergy™ HT Multi-Detection Microplate Reader. The Synergy HT is available with and without the optional twin reagent injector module.

Materials and Methods

Dual-Glo™ Luciferase Assay System (P/N E2920), Dual-Luciferase® Assay System (P/N E1910), and 5X Luciferase Passive Lysis buffer (P/N E1941) were purchased from Promega Corporation (Madison WI). Purified recombinant firefly enzyme (Quantilum®) was procured from Promega, while the recombinant *Renilla* luciferase (Novalite®) was from Chemicon (Temecula, CA). All experiments used Corning Costar 3912 white opaque microplates.

Both Dual Glo and Dual Flash luciferase assay kits were used in this study. Purified Firefly and *Renilla* luciferase enzymes were diluted independently to various concentrations. After dilution, 10 µl aliquots of Firefly and *Renilla* enzyme dilutions were pipetted into wells of a microplate that resulted in duplicate samples of a variety of molar ratios of the two enzymes in a total sample volume of 20 µl.

A Synergy HT with dispensers using well reading mode was employed for Dual- Luciferase® assay measurements. The reaction was initiated by the dispensing or 100 µl of reconstituted Luciferase Assay Reagent (LAR II) substrate by injector 1 into a well of the microplate. The luminescence of the well was then measured kinetically every 0.02 seconds over a period of 10 seconds. The PMT sensitivity was set at 100, which had previously been tested and shown to provide near-signal saturation by the highest concentration of Firefly luciferase. Following the completion of the read, 100 µl of Stop and Glo reagent was added using injector 2 (See Figure 3). This reagent terminates the Firefly luciferase signal and provides the substrate necessary for *Renilla* luciferase. The luminescent signal of the well was again measured kinetically every 0.02 seconds over a period of 10 seconds. After the completion of both injections and both reading periods, the plate was automatically moved by the reader and the process repeated on the next well until the entire plate had been read.

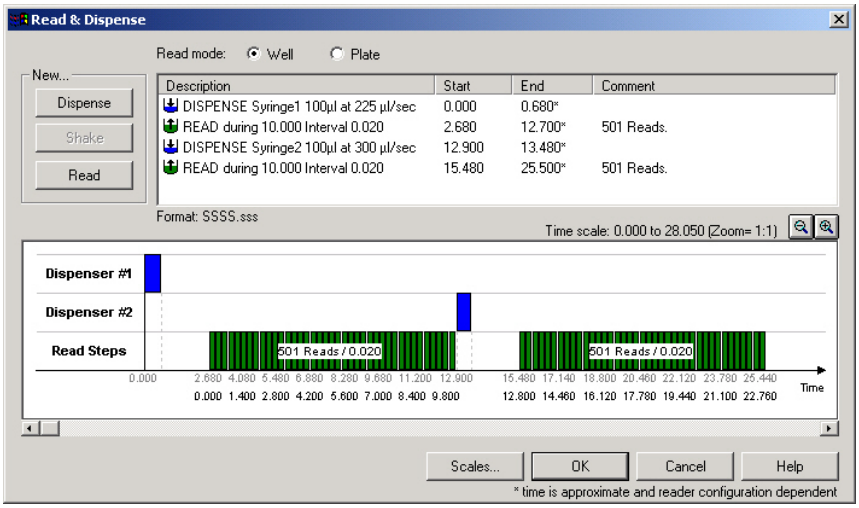


Figure 3. Dispense and Reading Parameters for Dual Luciferase Measurement. Flash luminescent assays such as Dual Luciferase, are read kinetically for a period of 10 seconds after the initiation of the reaction by the addition of substrate reagent.

For measurements using the Dual-Glo assay kit, each well then received 55 µl of 1X passive lysis buffer for a total sample volume of 75 µl. Next, 75 µl of Dual-Glo substrate was pipetted into each well of the microplate manually. The plate was then submitted to the Synergy HT reader and allowed to dark adapt at ambient temperature for 10 minutes. The luminescence of the plate was then measured using the Synergy HT. The PMT sensitivity was set automatically with the scale to high well software feature such that a known Firefly luciferase control was scaled to return a signal of 80,000. Following the completion of the read, 75 µl of Stop and Glo reagent was added manually and the plate read after a 10-minute wait for plate dark-adaptation. The luminescence was then measured as before, except the PMT sensitivity was scaled to 60,000 using a *Renilla* Luciferase control well. Data for both measurements were then exported to Microsoft® Excel for analysis.

Results

Figure 4 demonstrates an extreme example of the possible disparity that can be present in dual luciferase samples. Dilutions of Firefly and *Renilla* were pipetted into wells of a microplate in opposite directions, with the highest concentration of Firefly measured being present in the absence of *Renilla* and vice versa. This results in a molar ratio (Firefly/*Renilla*) ranging from 0 to infinity. In those samples that contain no Firefly, but large amounts of *Renilla* (i.e. very low Firefly/*Renilla* molar ratios), one observes an increase of *Renilla* activity greater than 5000 fold with the addition of Stop and Glo reagent to the samples. Similarly, samples that have very high molar ratios exhibit a tremendous quench in signal with the addition of Stop and Glo reagent. Equally important, is that in the absence of *Renilla* enzyme, no activity is observed in the presence of significant Firefly enzyme. This is further corroborated by the data presented in

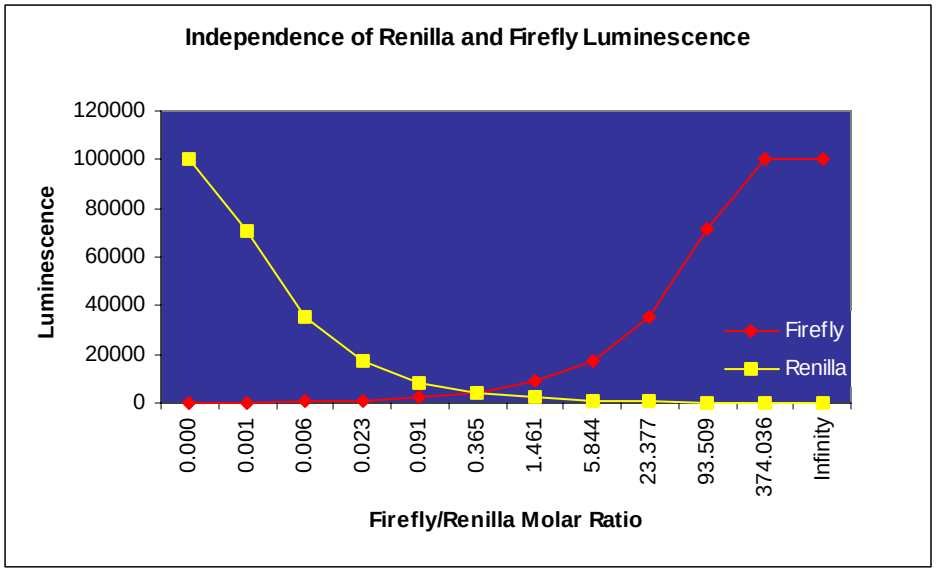


Figure 4. Firefly and *Renilla* Signal independence. The luminescence produced by Firefly and *Renilla* enzymes present in the same well at various molar concentrations were measured using a Synergy HT and the Dual-Luciferase® kit. The signal for each was measured and plotted independently as a function of the molar ratio of the two enzymes. Note that the data points represent the mean of duplicate determinations.

Table 1 presents the luminescence data obtained from 8 wells containing only Firefly luciferase after the addition of LAR II reagent (Firefly substrate) and the Stop and Glo (*Renilla* substrate). The average signal after the addition of LAR II reagent, which contains the substrate for Firefly luciferase, is in excess of 3,800,000 RLUs, whereas, the average signal in the same wells after the addition of the Stop and Glo reagent was 125 after subtraction of the blank well values (Table 1). This represents a 350,000-fold decrease or quench of signal as a result of the addition of the stop agent.

	Raw Data		Blanked Data		
Sample	LAR II	Stop & Glo	LAR II	Stop & Glo	Ratio
1	39316876	1178	39306278	123	319381
2	37940952	1242	37930354	187	202663
3	38103128	1159	38092530	103	368578
4	39486932	1118	39476334	63	627365
5	39036117	1220	39025519	164	237263
6	38725659	1152	38715061	97	399367
7	38785423	1151	38774825	96	404094
8	39254987	1218	39244389	163	241489
average	38831259	1180	38820661	125	350025
Blank	10598	1055			

Table 1. Firefly Luciferase Quenching with Stop and Glo Reagent. The blank value was determined from the average luminescent signal of two wells that do not contain Firefly or *Renilla* luciferase. This value was subtracted from each corresponding raw data value. Note that the ratio is based on the blank subtracted values.

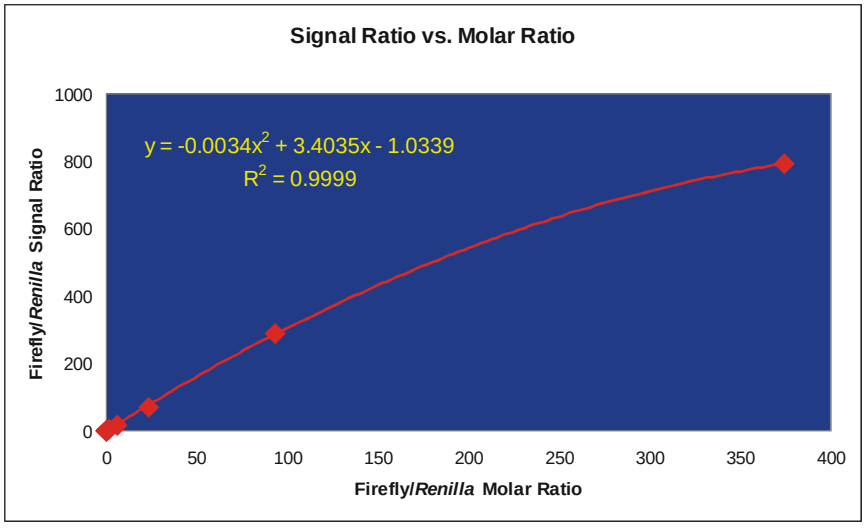


Figure 5. Signal Ratio Plotted against Molar Ratio. Dilutions of each enzyme (Firefly and *Renilla*) were pipetted into wells in various ratios and assayed. The ratio of the Firefly signal to the *Renilla* signal data was plotted as a function of the molar ratio of the two enzymes present. A Polynomial regression of the data was then performed using Microsoft® Excel. Note that each data point (diamond) represents the mean of replicate determinations.

When the relationship between the signal-ratio (Firefly/*Renilla*) to the molar ratio (Firefly/*Renilla*) is examined, a linear relationship is observed. Under conditions where a constant amount of *Renilla* is present, with changing amounts of Firefly luciferase (Figure 5), there is very good correlation between the signal to molar ratio. Likewise, under conditions of constant Firefly enzyme with changing amounts of *Renilla* in the same well, the relationship is linear. These data demonstrate that the two luminescent signals are independent of one another despite being in the same well.

Figure 6 demonstrates the consistency of signal of the reader. Different samples with the same enzyme concentration (Firefly or *Renilla*) exhibit the same luminescent response when assayed in parallel. The samples, while having the same concentration of measured enzyme, had very different molar ratios of the two enzymes. In these experiments, serial dilutions of enzyme were assayed in the presence of increasing, decreasing or constant amounts of the alternate enzyme. In either case (Firefly or *Renilla*) the returned signal was virtually identical.

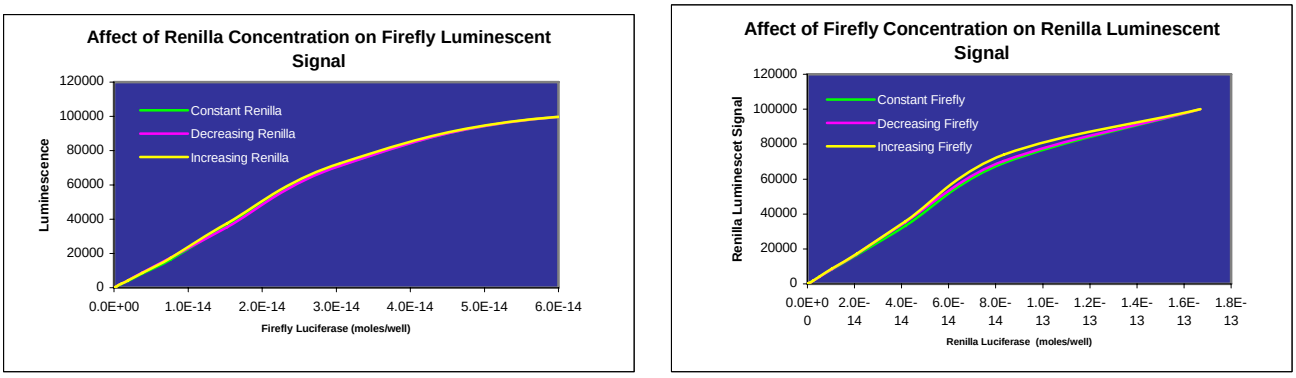


Figure 6. Consistency of Signal. Dilutions of each enzyme (A, Firefly and B, *Renilla*) were made independently and assayed sequentially in the presence of either a constant, increasing or decreasing amount of each other.

The results in Figure 7 demonstrate the utility of the Synergy™ HT Multi-Detection Reader to measure both Firefly and *Renill*a luciferase signals using a glow assay that does not require reagent injectors. The luminescence produced by Firefly luciferase levels ranging from 0 to 1.2 x 10^{-13} moles per well were assayed using the Promega Dual-Glo™ luciferase kit. Within the concentrations measured the response was found to be linear, with background levels averaging 25 RLU units at the PMT sensitivity setting used. The signal generated by 2.4 x 10^{-16} moles of Firefly luciferase was found to be significantly different than the blank using a signal/noise ratio of greater than 2 as the criteria (data not shown). When dilutions of *Renilla* luciferase were measured using the Dual-Glo kit, a linear relationship between the enzyme concentration and the luminescent signal was also observed. The substrate for *Renilla* luciferase is contained in the Stop and Glo reagent, which also inactivates any Firefly luciferase activity present. In our hands, the detection of *Renill*a luciferase was not as sensitive as Firefly luciferase on a molar basis, with a determined detection limit of 6.5 x 10^{-16} moles being significant.

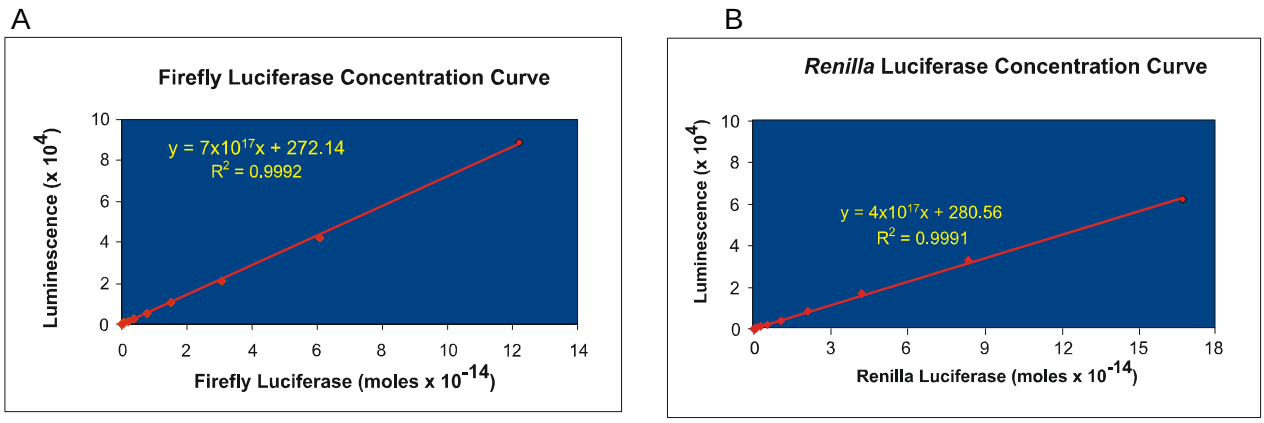
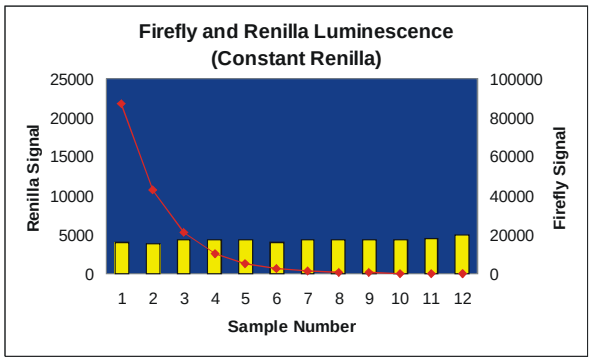


Figure 7. Firefly Luciferase and *Renilla* Luciferase Concentration curves. Dilutions of each enzyme (A, Firefly and B, *Renilla*) were made independently and assayed sequentially in the presence of a constant amount of each other. The corresponding signal for each was then exported to Microsoft Excel and linear regression analysis performed. Note that each data point (diamond) represents the mean of replicate determinations.

Measurements of the two enzymes can be made independently from one another despite having both activities present in the same sample. Figure 8 demonstrates the independence of the luminescence generated by the two enzymes. In either case, each sample contains both enzymes. In Figure 8A constant amounts of *Renilla* enzyme were present in all of the wells while the amount of Firefly luciferase varied dramatically and in Figure 8B constant amounts of Firefly were present with various concentrations of *Renilla*. Despite the tremendous excess of Firefly or *Renilla* signal present respectively in the samples, the constant luciferase signal is unaffected.

The measurement of Firefly and *Renilla* luciferase in the same sample is performed in order to provide an internal control. In other words, one of the luciferase activities is used for the experimental measurement, while the other signal is used to normalize the data between different samples. The process of normalization is most easily accomplished by calculating the ratio between the two activities. As demonstrated in Figure 9, when the data is expressed as a ratio, there is a linear relationship between the signal ratio (Firefly/*Renilla*) and the enzyme molar ratio (Firefly/*Renilla*). This indicates that the signal ratio can be used as a definitive means to normalize different samples.

A



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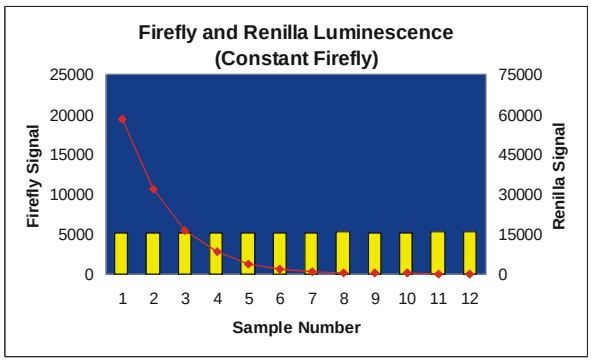

Figure 8. Firefly and *Renilla* signals from Common wells. Serial dilutions of Firefly and Renilla serial dilutions were aliquoted into wells of a microplate containing a constant amount of *Renilla* and Firefly enzyme respectively. Subsequent determinations of each (Firefly and *Renilla*) were made and the data plotted. Note that the data points for each represent the mean of duplicate determinations made from the same wells.

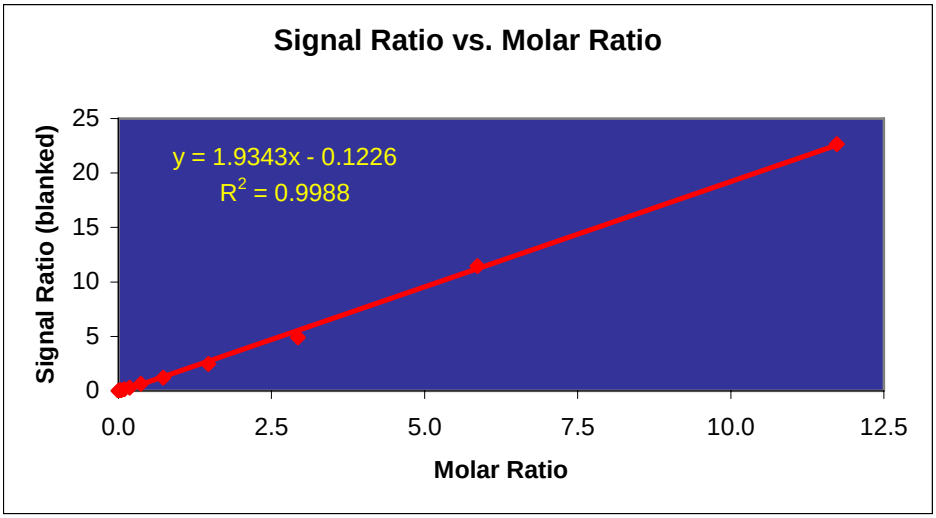

Figure 9. Comparison of the Firefly/*Renilla* Signal Ratio to Firefly/*Renilla* Enzyme molar ratio. The ratio of the Firefly signal to the *Renilla* signal data was plotted as a function of the molar ratio of the two enzymes present. Linear regression of the data point was then performed using Microsoft Excel.

Summary

- Synergy™ HT is capable of Performing Routine Luminescent Assays
  - Luciferase/ATP Assays
  - Dual Luciferase Measurements
    - Injector versions available for Flash Assays
    - Dual Glo Assay for HTS Applications

- Repeatable and Consistent Signal
- High Degree of Sensitivity
- Signal Independence
- Linear Signal
    - Enzyme Conc. vs. Signal
    - Molar Ratio vs. Signal Ratio
- Multi-Mode Detection
- Robotics Compatible

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