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Application Note

Optimizing Dual-Glo™ Luciferase Assays with the Synergy™HT Multi-Detection Microplate Reader

Introduction

Today's biological science and drug discovery research often involves the measurement of large numbers of samples. Sample replicates, experimental conditions or a large number of test compounds all add to the total number of measurements that need to be performed, regardless of the specific assay. One type of assay technology commonly employed is gene reporter assays. Genetic reporting assays are widely used to study gene expression and cellular responses to external stimuli in Prokaryotic and Eukaryotic organisms. Dual-reporter assays, as the name implies, use two independent reporter systems simultaneously to improve experimental accuracy. One reporter is usually tied to measuring the response resulting from the experimental conditions and is often referred to as the "experimental" reporter. The other 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-Glo™ Luciferase Assay System uses the activities of luminescent proteins (luciferases) from the firefly beetle (*Photinus pyralis*) and the sea pansy (*Renilla renformis*) to serve as an experimental and a control reporter respectively [1]. Here we describe the use of the Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments) to perform Dual-Glo™ luciferase measurements with purified recombinant enzymes. Promega's Dual-Glo™ luciferase assay system employs the sequential addition of two reconstituted reagents with luminescence measurement after each reagent addition. The first reagent, Dual-Glo Luciferase substrate provides the necessary chemistries and substrate for firefly luciferase, but does not contain the substrate for *Renilla* luciferase. Thus, luminescent measurements made after the addition of this reagent are entirely the result of firefly luciferase activity. The second reagent, Stop and Glo, contains proprietary chemistry that quenches the activity of firefly luciferase, while at the same time activating *Renilla* luciferase [1]. This system has been designed for the direct addition of the initial reagent to cell cultures (with media) without washing or preconditioning. Unlike flash luminescent assays, which require reagents added and measurements taken on a well-by-well basis (well mode), this procedure produces signals that

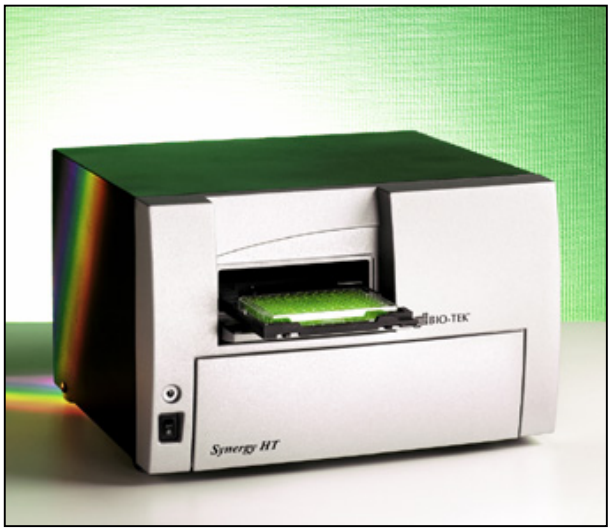

Figure 1. Synergy™ HT Multi-Detection Microplate Reader

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are stable over a long period of time. As a result, reagents can be added outside of the reader and luminescent determinations can be made on the entire plate (plate mode).

Materials and Methods

Dual-Glo™ Luciferase Assay System (P/N E2920) 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 recombinant *Renilla* luciferase (Novalite®) was from Chemicon (Temecula, CA). All experiments used Corning Costar 3912 white opaque microplates.

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. Each well then received 55 µl of 1X Passive lysis buffer for a total sample volume of 75 µl. Next, 75 µl of reconstituted Dual-Glo substrate was pipetted into each well of the microplate. Note that the Dual-Glo substrate was reconstituted according to the kit instructions immediately prior to use. The plate was then submitted to the Synergy™ HT Multi-Detection Microplate Reader and allowed to incubate at ambient temperature for 10 minutes. This incubation serves to allow for adequate reagent mixing, as well as allowing the microplate to dark-adapt. The luminescence of the plate was then measured using the Synergy HT. The PMT sensitivity was set automatically with the "Scale to high Well" feature Kineti-Calc Data Analysis Software (BioTek Instruments) such that a known Firefly luciferase control was scaled to return a signal of 80,000 RLU. Following the completion of the read, 75 µl of Stop and Glo reagent was added. This reagent, prepared immediately prior to use according to the kit instructions, terminates the firefly luciferase signal, and provides the substrate necessary for *Renilla* luciferase. Again, the plate was submitted to the Synergy HT reader for 10-minute ambient temperature incubation. The luminescence was then measured as before, except that the PMT sensitivity was scaled to 60,000 RLU using a Renilla Luciferase control well. Data for both measurements were then exported to Microsoft Excel for analysis.

Results

The results in Figure 2 demonstrate the utility of the Synergy™ HT Multi-Detection Microplate Reader to measure both Firefly and *Renill*a luciferase signals. The luminescence produced by Firefly luciferase levels ranging from 0 to 1.2×10^{-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 2.4×10^{-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 6.5×10^{-16} moles being significant.

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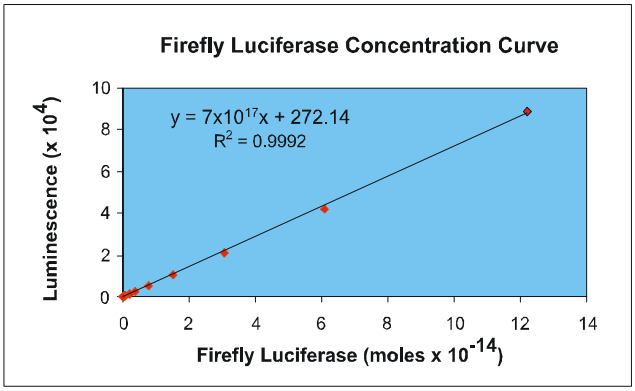

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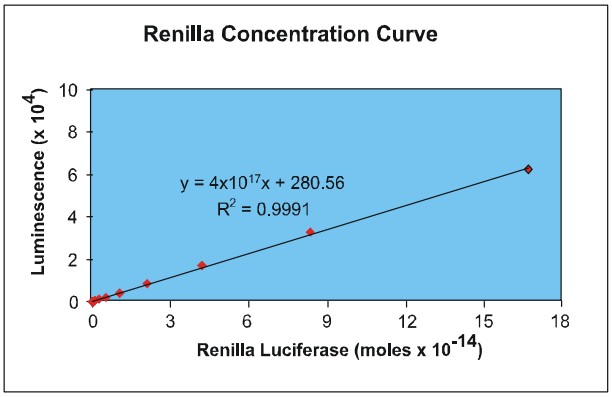


Figure 2. Firefly Luciferase and *Renilla* Luciferase Concentrations curves. Dilutions of each enzyme (A, Firefly and B, *Renilla*) were made independently and assayed sequentially in the presence of a constant amount of each other. The corresponding signal for each was then exported to Microsoft Excel 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 3 demonstrates the independence of the luminescence generated by the two enzymes. In each sample both enzymes were present, with constant amounts of *Renilla* enzyme in all the wells while the amount of Firefly luciferase varied dramatically. Despite the tremendous excess of Firefly signal present in three of the samples, the *Renilla* signal is unaffected. Likewise the presence of Renilla in sample 12, which does not contain any Firefly luciferase, does not increase the signal observed when that sample is assayed for Firefly luciferase activity.

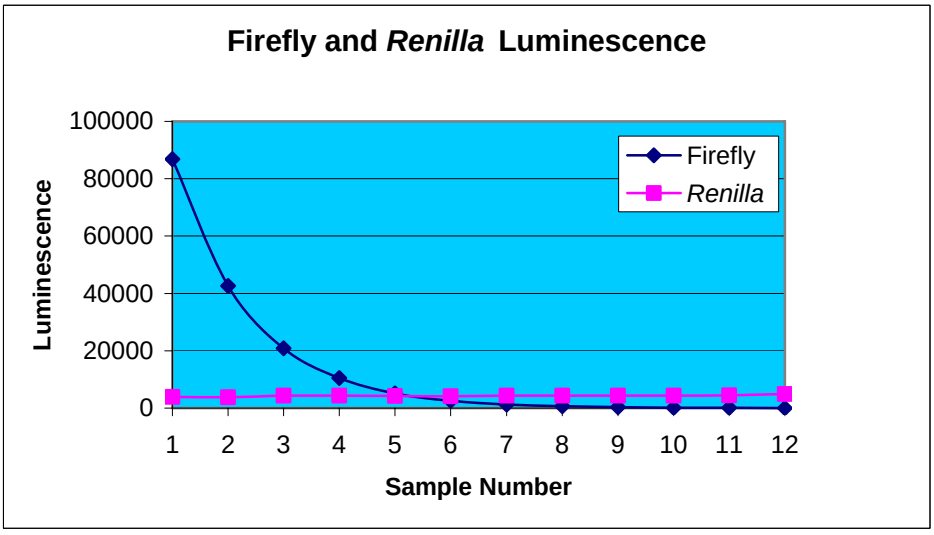


Figure 3. Firefly and *Renilla* signals from Common wells. Firefly serial dilutions were aliquoted into wells of a microplate containing a constant amount of *Renilla* enzyme. Subsequent determinations of each (Firefly and *Renilla*) were made and the data plotted. Note that the data points for each represent the mean of duplicate determinations made from the same wells.

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 4, when the data previously presented in Figure 3 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.

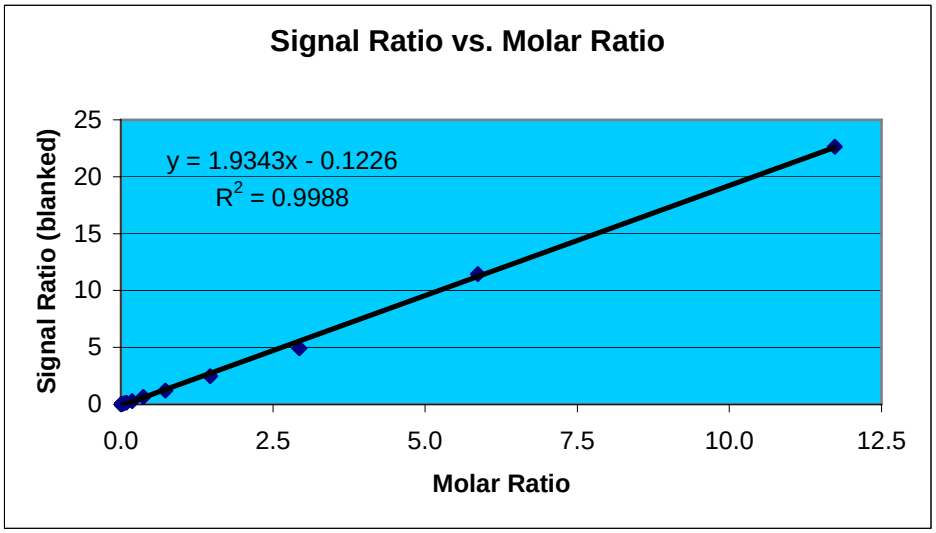
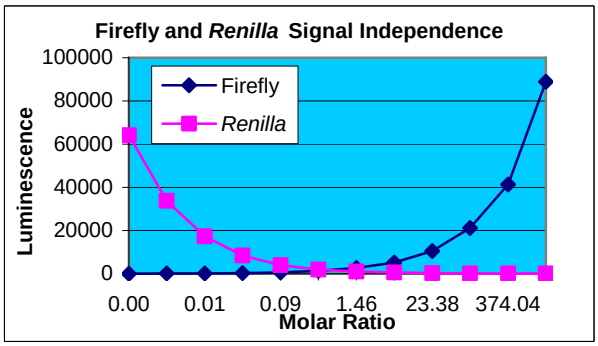


Figure 4. Comparison of the Firefly/*Renilla* Signal Ratio to Firefly/*Renilla* Enzyme molar ratio. The ratio of the firefly signal to the *Renilla* signal data presented in Figure 3 was plotted as a function of the molar ratio of the two enzymes present. Linear regression of the data points was then performed using Microsoft Excel.

Figure 5 demonstrates an extreme example of the possible disparity that can be present in 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. When these data are expressed as a signal ratio versus a molar ratio and plotted, a linear relationship is observed. These data are very similar to that seen in Figure 4 up to a molar ratio of approximately 90. The samples with higher molar ratios were those that had very low concentrations of *Renilla* enzyme. These dilutions resulted in a signal that was below the detection limit of the assay and in a loss of linearity leading to a polynomial relationship (data not shown). Despite the discrepancy at very high molar ratios these data demonstrate that the signal ratio can be used quantitatively to compare samples.

A



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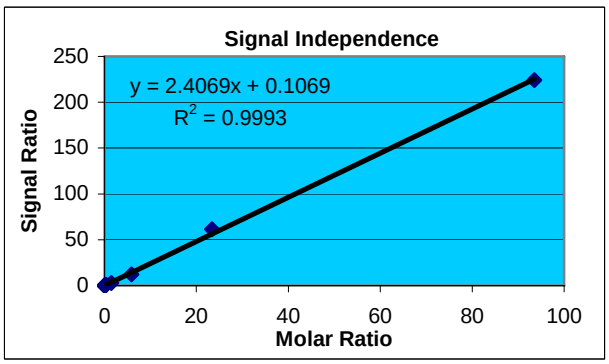

Figure 5. Firefly and Renilla Signal independence. The luminescence produced by Firefly and *Renilla* enzymes present in the same well at various molar concentrations were measured using a Synergy HT and the Dual-Glo Assay kit. (A) The signal for each was measured and plotted independently as a function of the molar ratio of the two enzymes. (B) The ratio of the two signals (Firefly/*Renilla*) was plotted against the molar ratio of the two enzymes and a linear regression analysis performed. Note that the data points represent the mean of duplicate determinations.

Discussion

These data demonstrate that the Synergy™ HT Multi-Detection Microplate Reader is capable of performing the luminescent measurements required for the Dual-Glo luciferase assay system. The Synergy HT Multidetection Reader is a robotic compatible microplate reader that can measure absorbance, fluorescence, and luminescence in all plate formats up to 384-well plates (Figure 1). 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. Emitted luminescence is captured using either the top or bottom probes and the light measurements obtained using the Synergy HT's low noise PMT operated in photon integration mode. In addition to its optical attributes, the Synergy HT reader has numerous features that enhance the reader's capability. Elevated temperatures are regulated by a four-zone microprocessor-controlled system that assures superior temperature uniformity up to 50°C. With a compact footprint, a robot friendly carrier design, USB and RS-232 connection, the Synergy HT is also compatible with many of the commonly preferred robotics systems. The functionality of the Synergy HT is greatly enhanced by its controlling data reduction software, KC4.

The Dual-Glo™ assay is ideal for experimental situations where large numbers of gene reporter samples are required. Both the Firefly and the *Renilla* luminescent signal are stable over a long period of time, eliminating the need for precise timing as it pertains to samples which are measured relative to the time the substrate was added. As long as the samples are all measured in a timely fashion relative to one another, the data will be legitimate. This allows for the pipetting processes to be carried out using separate pipetting devices to transfer the substrate reagents. The ability to perform the pipetting and reading on the entire plate at one time increases the efficiency of the assay tremendously. The Dual-Glo assay kit does sacrifice slightly in regards to detection of the firefly luciferase. The flash measurement is reported to be more sensitive than the equivalent glow measurement. However for most situations, particularly screening assays where large numbers of samples are being measured, the absolute best assay sensitivity is not required.

References

[1] Dual-Luciferase Technical Manual, Part Number TM058, Promega Corporation, 2800 Woods Hollow Rd. Madison, WI 53711-5399

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