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Application Note

Using the Synergy™HT Multi-Detection Microplate Reader to Run the Dual-Luciferase® Reporter Assay System

Introduction

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 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 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 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 a Synergy™ HT Multi-Detection Microplate Reader (BioTek Instruments) configured with injectors to perform dual-luciferase measurements with purified recombinant enzymes.

Firefly luciferase is a monomeric 61 kD enzyme that catalyses a two-step oxidation of luciferin, which yields light at 560 nm. The first step involves the activation of the protein by ATP to produce a reactive mixed anhydride intermediate. In the second step, the active intermediate reacts with oxygen to create a transient dioxetane, which quickly breaks down to the oxidized product oxyluciferin and carbon dioxide along with a burst of light [2]. *Renilla* Luciferase is a 31kD monomeric enzyme that catalyses the oxidation of coelenterazine to coelenteramide, also yielding carbon dioxide and blue light centered on 480 nm [3].

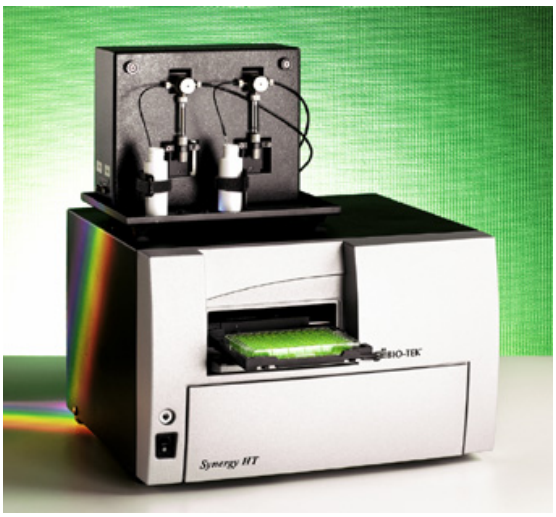

Figure 1. Synergy HT with Injectors.

Materials and Methods

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 recombinant *Renilla* luciferase (Novalite®) was from Chemicon (Temecula, CA). All experiments used Corning (Acton, MA) white opaque microplates (P/N 3912).

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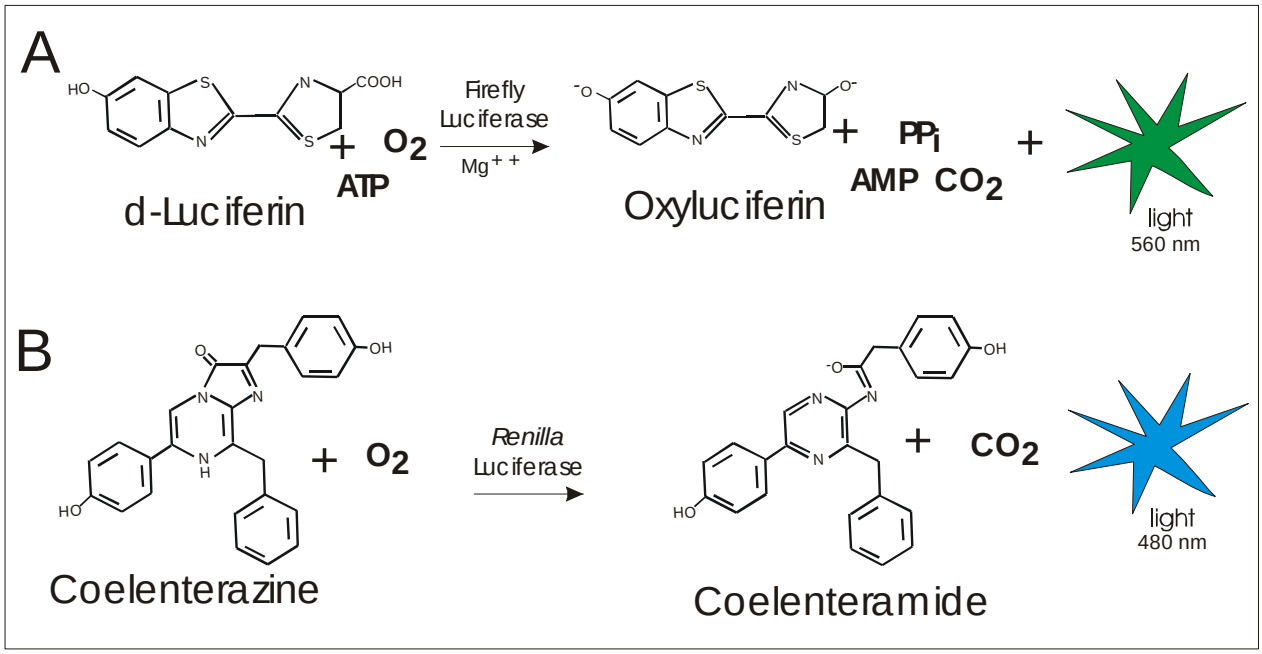


Figure 2. Bioluminescent Reactions Catalyzed by Firefly and *Renilla* Luciferase. (A) Firefly luciferase, using ATP, catalyses the two-step oxidation of luciferin to oxyluciferin, which yields light at 560 nm. (B) *Renilla* luciferase catalyses the oxidation of coelenterazine to coelenteramide, which yields light at 480 nm.

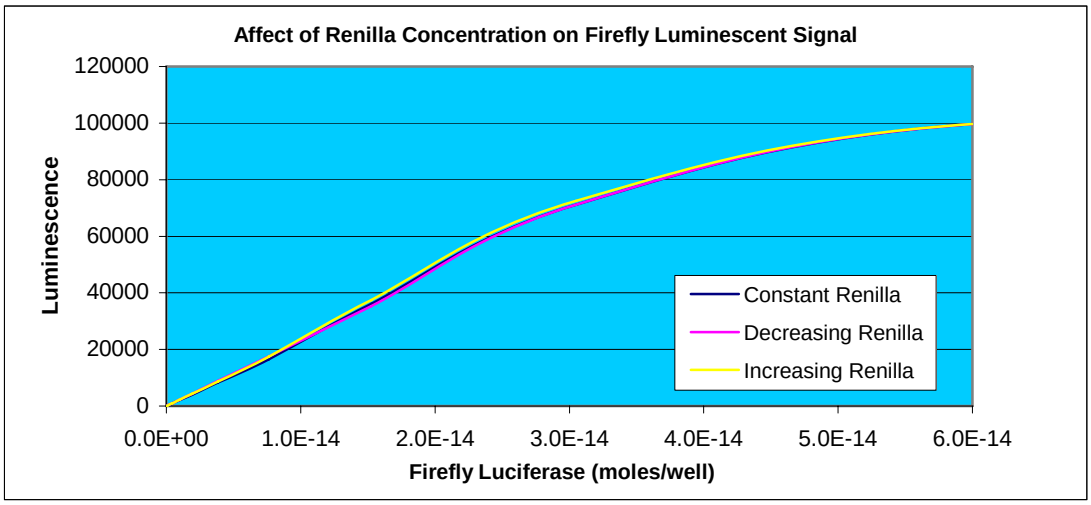
Purified Firefly and *Renilla* luciferase enzymes were diluted independently to various concentrations. After dilution, 10 µl aliquots of Firefly and Renilla enzyme dilutions were pipetted into wells of a microplate that resulted in duplicate samples of a variety of molar ratios of the two enzymes in a total sample volume of 20 µl. The plate was then submitted to the Synergy™ HT Multi-Detection Microplate Reader and allowed to dark adapt at ambient temperature for 10 minutes. This dark adaptation period allows the microplate to dissipate any residual autoluminescence, resulting from energy absorption by the plate itself. The plate was then read using a Synergy HT with Dispensers using well mode reading. The reaction was initiated by dispensing 100 µl of reconstituted Luciferase Assay Reagent (LAR II) substrate with 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 of the Synergy Reader. 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 automatically moved by the reader and the process repeated on the next well until the entire plate had been read. Data for both measurements were then exported to Microsoft Excel for analysis.

Results

In order for the dual luciferase assay system to function as required, the activity of each enzyme and, more importantly the signal returned from each enzyme activity, needs to be independent of one another despite both being present in the same well. When dilutions of Firefly luminescent activity were examined in the presence of increasing, constant, or decreasing concentrations of *Renilla*, the luminescent output was unaffected. As demonstrated in Figure 3A, the luminescent response of various concentrations of Firefly luciferase is virtually the same regardless of the

relative amount of Renilla enzyme present. Likewise, the signal generated by Renilla, as measured after the addition of Stop and Glo reagent, was found to be independent of the relative amount of Firefly enzyme present (Figure 3B).

A



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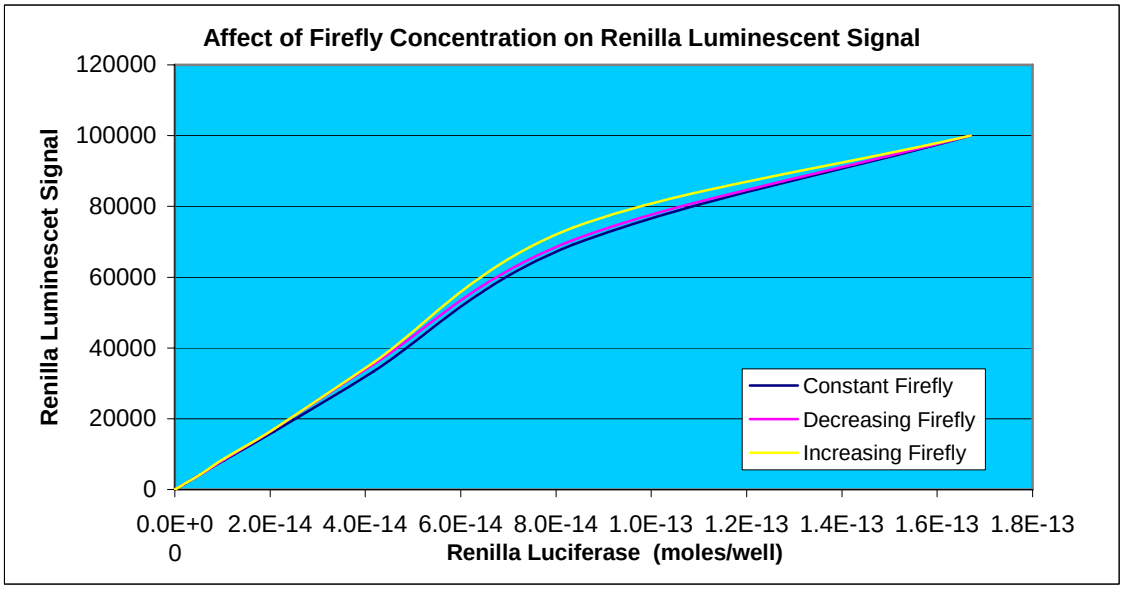


Figure 3. Firefly and Renilla Concentration curves. Dilutions of each enzyme (A, Firefly and B, *Renilla*) were made independently and assayed sequentially in the presence of either a constant, increasing or decreasing amount of each other. The corresponding signal for each was then exported to Microsoft Excel and plotted.

Figure 4 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. 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.

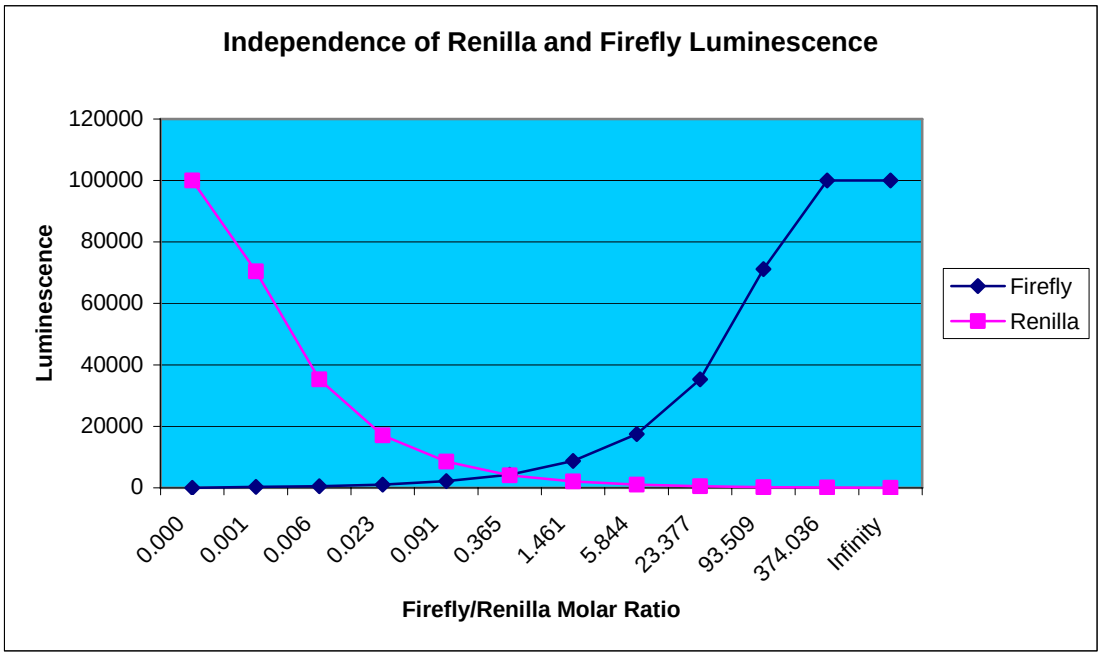


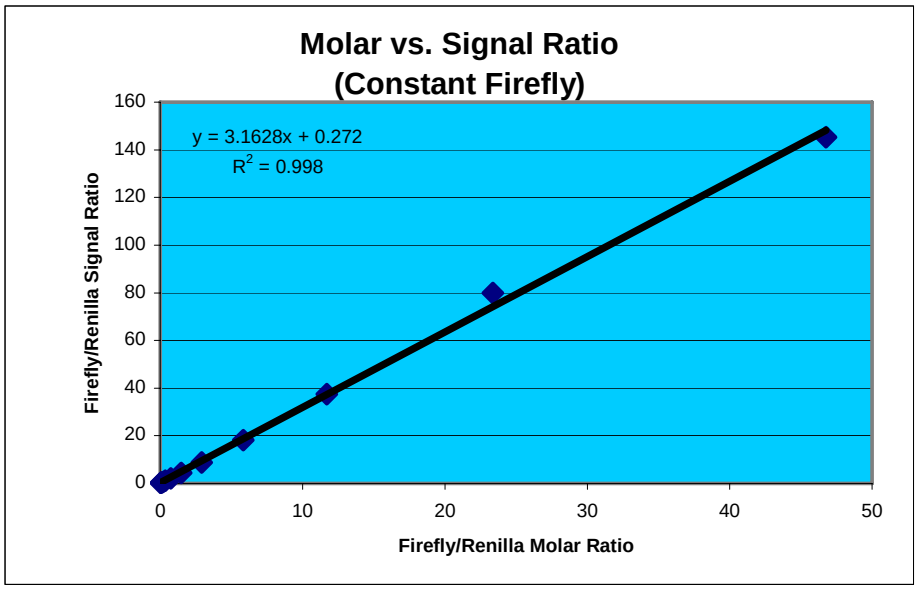
Figure 4. Firefly and Renilla Signal independence. The luminescence produced by Firefly and *Renilla* enzymes present in the same well at various molar concentrations were measured using a Synergy HT and the Dual-Luciferase kit. The signal for each was measured and plotted independently as a function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

	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.

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 Table 1. Which 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 RLU's 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.

A



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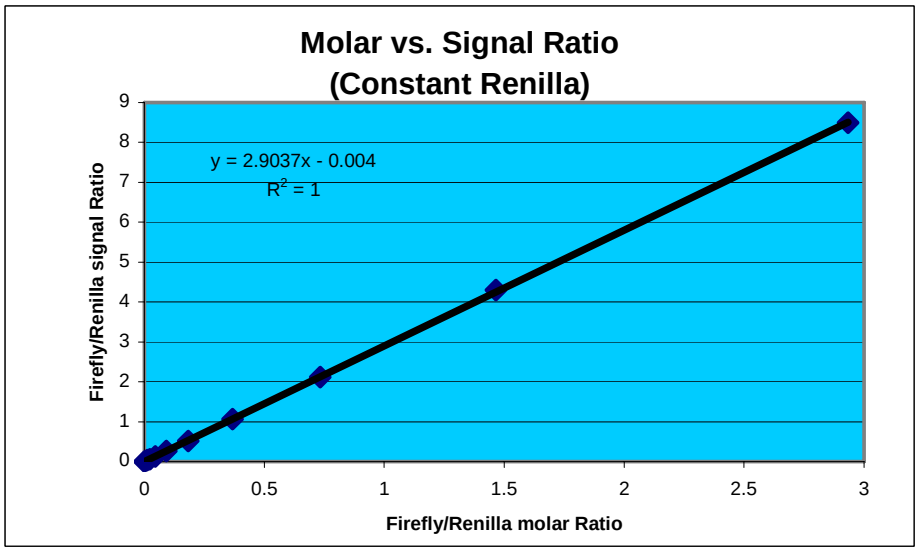

Figure 5. Firefly Luciferase and *Renilla* Luciferase Concentrations curves. Dilutions of each enzyme (A, Firefly and B, *Renilla*) were made independently and assayed sequentially in the presence of a

constant amount of each other. The 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. Note that each data point (diamond) represents the mean of replicate determinations.

When the relationship between the signal-ratio (Firefly/*Renilla*) and 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 5A), 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 as well (Figure 5B). These data demonstrate that the two luminescent signals are independent of one another despite being in the same well.

	Firefly Signal		Renilla Signal	
Sample	Mean	%CV	Mean	%CV
1	19613592	1.42	119495.1	2.84
2	2367610	6.41	8269051	1.48

Table 2. Signal Consistency. Two different samples, each containing Firefly and Renilla enzyme, but in different ratios were assayed for activity for each enzyme in replicates of eight. The mean value for all eight replicates as well as the %CV for both Firefly and Renilla signals were then calculated.

In order for measurements to be quantitative, they must be consistent and repeatable within the same experiment. As demonstrated in Table 2, replicates of the same sample return very similar results. On average, the CVs for two different samples containing both Firefly and *Renilla* were approximately 3%. While one sample's Firefly signal had a %CV of 6.41%, the remaining measurements were much more precise, suggesting that pipetting error was most likely the cause rather than the reader. In addition to being precise, the signals are stable over a period of time. Due to the length of time required to measure all the samples of a 96-well microplate it is important that the signal generated by the reader be stable, and that the reagents not interact in any deleterious way with the fluid path. When the same samples are measured 30 minutes apart, using the same reagents that have not been removed from the reader, very little differences are observed (Table 3). This demonstrates that samples measured at the beginning of the plate can be reliably related to those measured at the end.

Reading	Before	After	% Diff
Firefly Signal	19373838	18875001	2.57
Renilla Signal	115932	112417	3.03
Ratio	167.13	167.92	0.47

Table 3. Signal stability. A representative sample containing both Firefly and *Renilla* activity was assayed both before and after a 30-minute delay in replicates of eight. The difference in signal between the two measurements was compared. During the delay, the necessary reagents, (LAR II and Stop and Glo) remained in the fluid lines, while the enzyme sample remained on ice.

Discussion

These data presented indicate that the Synergy™ HT Multi-Detection Microplate Reader with injectors is capable of performing Promega's Dual-luciferase assay kit. This assay kit requires the injection of two different reagents followed by luminescent measurements after each. Because these reagents will interact with one another it is important that separate fluid paths be used. The Synergy HT Multidetection reader with injectors reader is configured with two completely separate injectors. Each of these injectors can be configured independently using KC4 software, with both being available for use within a single reading protocol. Multiple reading

windows are then used to make independent luminescence determinations for the two enzymes after each injection. This functionality makes the Synergy ideal for Dual-luciferase measurements.

Dual luciferase assays are employed in order to correct for subtle differences between experimental samples. One luciferase is used as the experimental reporter (usually firefly), while the other (*Renilla*) is used as an internal control to normalize data, often expressed as a ratio. The linear relationship between the signal and molar ratio indicates that the Synergy HT will provide faithful results when cell lysates are assayed.

There are several critical issues that the user needs to be cognizant of when running dual-luciferase assays. Proper instrument maintenance is a critical component in obtaining good results when performing the Dual-luciferase assay. Prior to a luminescent assay new run, it is suggested that all of the storage fluid (i.e. deionized water or 70% ethanol) present in the line be removed prior to priming. This will prevent any dilution or contamination of reagent during priming. The Stop and Glo® reagent used in the dual-luciferase assay has a reversible affinity to some types of plastics often used in injector systems. In order to insure that the residual reagent has been removed, it is recommended that the syringe and tubing be filled with 70% ethanol for approximately 30 minutes. Failure to properly clean this reagent after use can result in the reagent leaching back into the tubing with subsequent use. Following treatment with ethanol it is recommended that the injectors be rinsed with deionized water thoroughly to remove traces of ethanol. Contamination of the fluid path, particularly the injector tip by other assay reagents can often lead to aberrant results. Proper cleaning after use usually prevents this, but occasionally the replacement of the fluid path and injector tip may be necessary. The easy access of these items with the Synergy HT makes changing them straight forward.

References

1. Dual-Luciferase Technical Manual, Part Number TM046, Promega Corporation, 2800 Woods Hollow Rd. Madison, WI 53711-5399
2. de Wet JR, Wood KV, Helinski DR, DeLuca M, (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in Escherichia coli, Proc. Natl. Acad. Sci USA 82:7870-7873.
3. Matthews, J.C., Hori, K., and Cormier, M.J. (1977) Purification and Properties of *Renilla reniformis* Luciferase *BioChemistry*, 16:85-91.

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