



Using the Clarity™ Luminescence Microplate Reader to Perform Dual-Luciferase® Assays

Abstract

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 entails the use of 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 such that it does not respond to the experimental conditions, acting as an internal control from which data generated by the experimental reporter can be normalized to. 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 reniformis*) to serve as an experimental and a control reporter, respectively. Here we describe the use of the Clarity™ Luminescence Microplate Reader (Bio-Tek® Instruments) to perform dual-luciferase measurements with purified recombinant enzymes.

Introduction

Bioluminescence is a naturally occurring phenomenon that has been utilized for a number of applications, particularly in molecular biology where the associated enzymes have been used as genetic reporters. Bioluminescence is nearly ideal for use as a genetic marker. Typically there is no endogenous luminescent activity in somatic cells, while the experimentally introduced bioluminescence is nearly instantaneous, sensitive and quantitative. While numerous species exhibit bioluminescence, only a relative few different species have been characterized and cloned. Of these, only Firefly luciferase, *Renilla* luciferase and Aequorin have had much utility [1].



Figure 1. Clarity™ Luminescence Microplate Reader with two injectors.



Figure 2. Clarity's Removable Injector Tip. Injector tips are disposable and can easily be accessed and replaced if necessary.

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]. The utility of *Renilla* luciferase is somewhat limited by the presence of a low level of non-enzymatic autoluminescence, which reduces the assay sensitivity. While not as sensitive as Firefly

luciferase, which does not suffer from autoluminescence, *Renilla* luciferase has been used with good success in conjunction with Firefly luciferase as a reporter internal control to compensate for experimental variables such as transfection efficiency.

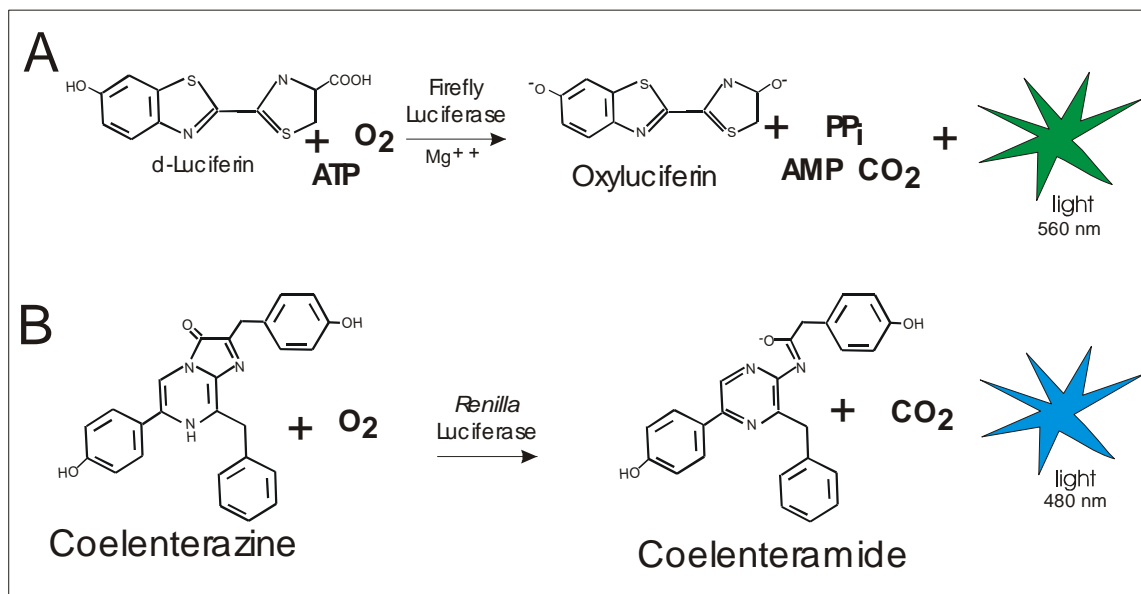


Figure 4. Bioluminescent Reactions Catalyzed by Firefly and *Renilla* Luciferase. (A) Firefly luciferase, using ATP, catalyses the two-step oxidation of luciferin to oxyluciferin, which yields light at 560 nm. (B) *Renilla* luciferase catalyses the oxidation of coelenterazine to coelenteramide, which yields light at 480 nm.

Promega's Dual-Luciferase® Reporter Assay System employs the sequential addition of two reconstituted reagents with luminescence measurement after each reagent addition. The first reagent, Luciferase Assay Reagent II (LARII) 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 [4].

The Clarity™ Luminescence Microplate Reader has been specifically designed for the detection of chemi- and bioluminescence. It can be employed for all measurements of glow and flash luminescence in 96- or 384-well microplates. The luminometer utilizes high precision reagent injectors in combination with an ultra sensitive photon counting photomultiplier tube (PMT) detector, which are controlled using external PC software. The luminometer is available with up to four reagent injectors, two of which are intimately associated with the detector. Each injector uses microprocessor-controlled syringes to deliver exact amounts from 10 to 150 µl of reagent through chemically inert tubing to a disposable injector tip adjacent to the detector. Three different modes (linear, orbital, and cross) are available for shaking of microplates.

The luminometer comes with the Clarity™ software package, which runs on the Microsoft® Windows® operating system. The software is structured in the form of protocols, allowing automation of all steps from defining measurement parameters to reporting final results. Clarity supports the following protocol types: Raw Data, Fast Kinetics, Dual Measurement and Batch Protocol. Users can create custom protocols for immediate use or store them for later availability. The Clarity protocol interface allows users to modify parameters such as injection volume, delay time and measurement duration. The software easily formats to interchange 96- and 384-well microplates. Any combination of wells can be read. For detailed data analysis, Bio-Tek's KC4™ Data Analysis Software can be used to perform data reduction and analysis.

The luminometer is ideal for the bench top, with a footprint of 15.4" (W) x 16.4" (D) and a height of 10.2" (38.5 x 41.0 x 25.5 cm respectively). Despite its compact design it has a durable design and a weight of 22 kgs. Clarity has a robotic friendly plate carrier and can be integrated into robotic systems using technical documentation provided. The luminometer offers both RS-232 and USB serial ports for PC communication.

Materials and Methods

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.

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 Clarity™ Luminescence Microplate Reader in dual measurement mode, 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 the 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.



Figure 3. Close up of two injector syringes and reagent bottles. Clarity can be provided with up to 4 syringe injectors. Note that each injector has a freely movable reagent bottle holder, inert tubing and a dedicated syringe to control injection volume.

Results

Using purified recombinant Firefly and *Renilla* luciferase enzymes the ability of the Clarity reader to perform dual luciferase assays was assessed. The ability of the reader to specifically identify the individual luciferase enzymes with the Dual Luciferase kit is demonstrated in Figure 5. Virtually no *Renilla* activity is observed in the absence of the enzyme, while significant Firefly signal is present. Equally important is that in the absence of Firefly enzyme no activity is observed in the presence of significant *Renilla* enzyme. This is further corroborated by the data presented in Tables 1 and 2. Table 1 presents the luminescence data obtained from 16 wells containing only Firefly luciferase after the addition of LARII 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 9,100,000 RLUs, whereas, the average signal in the same wells after the addition of the Stop and Glo reagent was 550 (Table 1).

Firefly Luciferase Quenching with Stop and Glo Reagent			
Sample	LAR II	Stop and Glo	Ratio#
1	8766006	424	22402
2	8985109	603	15754
3	8985652	357	27708
4	8923102	454	21180
5	8927876	381	25633
6	9072594	598	16048
7	9039405	452	21558
8	8906707	323	30682
9	8997296	707	13342
10	9239756	416	24106
11	9344078	727	13458
12	9337985	840	11566
13	9288531	441	22749
14	9284649	815	11868
15	9300746	360	28417
16	9227987	327	31357
		Average	21114

Note that blank values of 1204 and 33 for the LAR II and Stop and Glo respectively were subtracted from each data point prior to ratio calculation.

Table 1. Firefly Luciferase Quenching with Stop and Glo Reagent.

Similarly, the signal in eight wells containing only *Renilla* luciferase increased from an average of 2366 RLUs after the addition of LAR II reagent to over 6,100,000 RLUs with the addition of Stop and Glo Reagent. This represents a greater than 5000 fold increase in signal after the subtraction of the appropriate blank values from each measurement (Table 2).

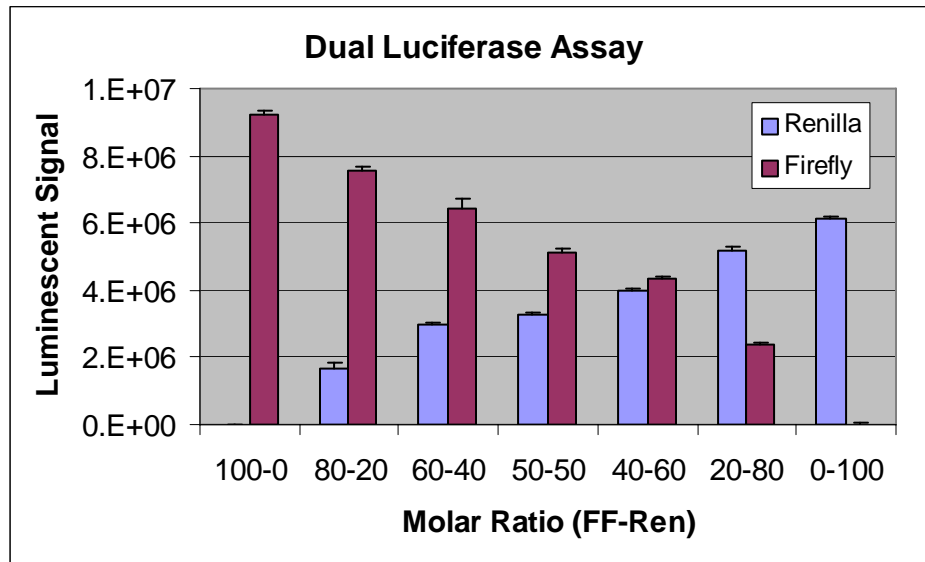


Figure 5. Signal of Firefly and *Renilla* Luciferase when measured in a Dual Luciferase Assay. Dilutions of Firefly and *Renilla* luciferase enzyme were assayed alone or in combination using a Dual Luciferase Kit (Promega, Madison, WI). Using approximately equal concentrations different volumes of each enzyme were pipetted into microplates in replicates of 8. Each bar represents the mean of 8 determinations.

The rate at which the reagents are dispensed to the wells can be critical. By controlling the fluid dispense-rate problems associated with the assay can be avoided. The kit manufacturer recommends a slower rate when dispensing reagent 1 (LAR II), as it avoids splashing the sides of the well, which has the potential to leave a thin film of luminescent solution that can escape mixing when the Stop and Glo reagent is added later [3]. The subsequent addition of Stop and Glo needs to be at a faster rate to insure rapid mixing of the reagents. Rapid mixing is required due to the necessity of terminating the Firefly luciferase activity prior to the initiation of *Renilla* luciferase measurement. The kit manufacturer suggests that at a minimum, the ability to achieve a 10,000 fold quenching is necessary [4]. Table 1 demonstrates an average of greater than a 20,000-fold quenching can be achieved using the Clarity™ Luminescence Microplate Reader.

In experimental situations, both Firefly and *Renilla* activities will be present in the well at the same time. It is important that both activities not influence the other's signal to any extent. As demonstrated by Figure 5, when different ratios of firefly and *Renilla* are present in the same well each signal is in proportion to its own concentration, as the Firefly signal tracks its own concentration as does *Renilla*, despite having the same total number of moles of luciferase in each well. In addition the error bars for each luciferase remain relatively unchanged despite increasing amounts of the other. When these data can be plotted independently and a linear regression analysis performed a significant correlation between signal and enzyme concentration is observed (Figure 6). The difference in total signal between Firefly and *Renilla* suggests that true active enzyme concentrations of the two enzymes might have been different, with Firefly being more active. Figure 7 demonstrates the relationship between molar ratio and signal ratio. As noted by the least means squared linear regression equation, a very close 1:1 relationship exists. This close relationship between the two is important due to the nature in which these two enzymes are used experimentally. In order for *Renilla* to be effectively used as an internal control and normalize the experimental results obtained with the Firefly luciferase, the response signal needs to track the molar amounts present in the lysates.

Renilla Activity with and without Stop and Glo Reagent			
Sample	LAR II	Stop and Glo	Fold Increase [#]
1	2077	6016578	6893
2	2418	6176637	5088
3	2428	6160230	5033
4	2396	6203670	5205
5	2413	6128166	5069
6	2396	6065448	5089
7	2401	6051479	5056
8	2402	6251840	5219
		Average	5332

Note that mean blank values of 1204 and 33 for the LAR II and Stop and Glo respectively were subtracted from each data point prior to fold increase calculation.

Table 2. Renilla Activity with and without Stop and Glo Reagent.

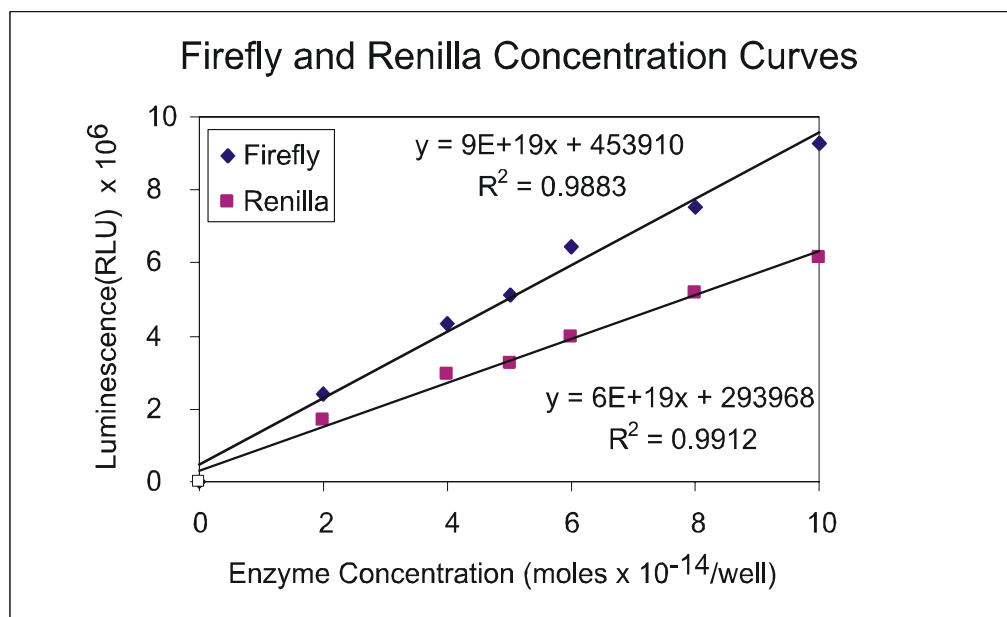


Figure 6. Firefly and Renilla Luciferase Concentration Curves. Mixtures of purified Firefly and *Renilla* luciferase enzymes were assayed using a Dual-Luciferase® kit, with luminescence measured with a Clarity™ Luminescence Microplate Reader.

Discussion

These data presented indicate that the Clarity™ Luminescence Microplate Reader is versatile and capable microplate luminometer 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. Clarity™ can be configured with two completely separate injectors (Figure 3), use each injector within a single reading protocol, as well as having the capability of making independent luminescence determination after each injection. This functionality makes the Clarity™ 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 Clarity™ Luminescence Microplate Reader will provide faithful results when cell lysates are assayed.

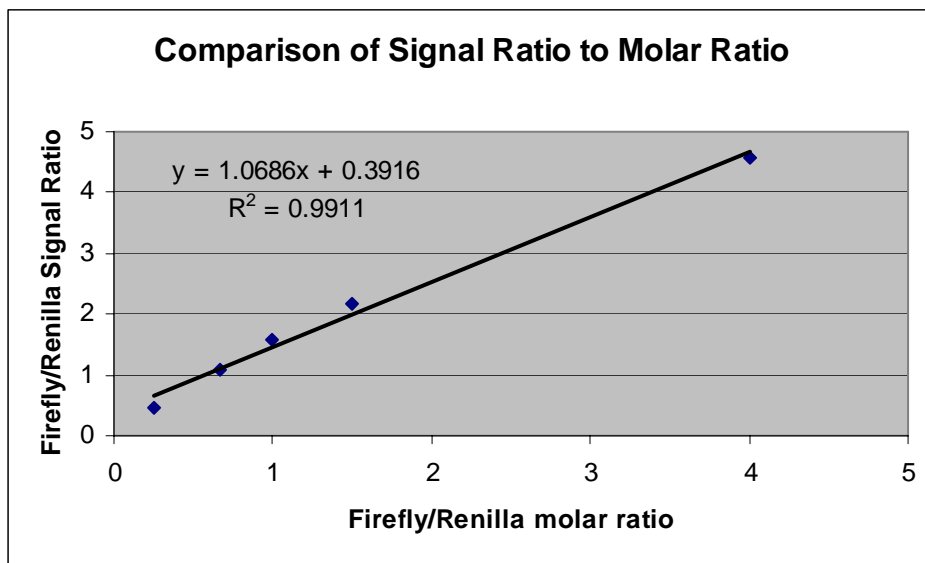


Figure 7. Comparison of Firefly to *Renilla* signal ratio to molar ratio. The ratio of Firefly to *Renilla* luminescent signal was plotted against the molar ratio of the two enzymes at 5 different ratio values. Diamonds represent the mean of 8 determinations.

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. While the Clarity™ Luminescence Microplate Reader uses tubing similar to Teflon®, which does not suffer from reagent affinity, other luminometers have been known to use Tygon® tubing, which may require soaking overnight (16 hours) to completely remove residual reagent. 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 Clarity™ Luminescence Microplate Reader (Figure 2) makes changing them straight forward.

Background signal is usually present and caused by a number of different factors. Instrument electronic noise is a common cause of background. The Clarity™ Luminescence Microplate Reader has the ability to perform a measurement on a dark chamber prior to the initiation of the assay and subtract the signal from each measurement. During a Dual-Luciferase® measurement, Firefly luciferase does not normally exhibit luminescence in the absence of substrate. However, microplates have been known to exhibit background luminescence from a number of different causes. Polystyrene has the capacity to accumulate significant amounts of static electrical buildup, the discharge of which can result in elevated backgrounds. In addition, microplates are prone to phosphorescence. They absorb energy from overhead lights, particularly fluorescent lights, during the preparation of the luminescent assay, then slowly emit light energy when they are in the dark reading chamber of the luminometer. In addition to the previously mentioned causes, background luminescence during *Renilla* luciferase measurements can result from the autoluminescence of the coelenterazine substrate or from residual luminescence from the Firefly reaction. These backgrounds can be accounted for by using appropriate reagent only control wells and subtracted from the experimental data prior to ratio calculations.

References

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