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Luciferase Measurements using the Clarity™ Luminescence Microplate Reader

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Abstract

Reporter genes, such as luciferase, are important tools for studying gene expression. The use of reporter genes allows the *in vitro* and *in vivo* measurement of gene expression from virtually any endogenous genetic control element. Luciferase enzyme and its subsequent luminescent reaction is often the gene reporter of choice for many experimental conditions. Here we describe the measurement of luciferase enzyme luminescent activity using the Clarity™ Luminescence Microplate Reader.

Introduction

Bioluminescence is a naturally occurring phenomenon that has been utilized for a number of applications, particularly in molecular biology where the enzyme associated with it 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 have been characterized and cloned. Of these, only Firefly luciferase, *Renilla* luciferase and Aequorin have had much utility.

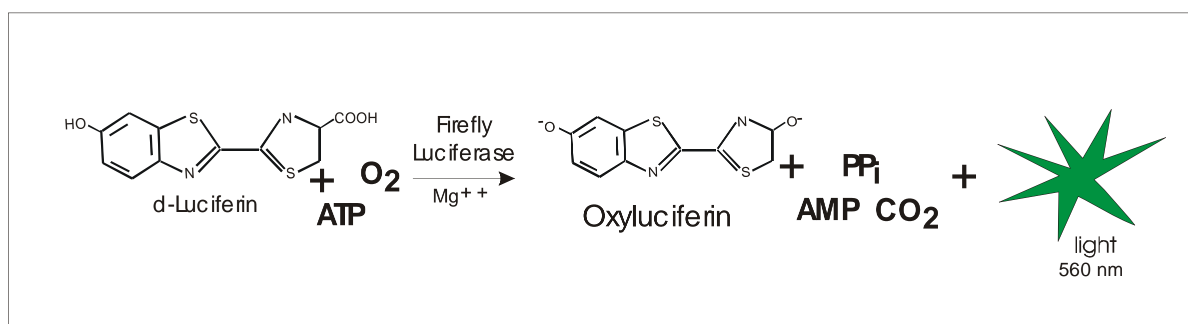

Figure 1. Bioluminescent Reactions Catalyzed by Firefly Luciferase. Firefly luciferase, using ATP, catalyses the two-step oxidation of luciferin to oxyluciferin, which yields light at 560 nm.

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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 [1]. Luciferase has many characteristics that make it ideal for a reporter. Its activity is not dependent on any post-translational modification, making it immediately available for quantitation. In addition, the luminescent is very bright, having very high quantum efficiency as compared to many other chemiluminescent reactions [2].

Conventional “flash” type assays using Firefly luciferase result in a flash of light, which decays rapidly with the addition of substrates to the enzyme. The Promega kit used incorporates coenzyme A (CoA) to increase enzyme turnover, resulting in an increased light output that is maintained for at least 60 seconds [3]. While the addition of CoA has been used to stabilize the light output for the reaction, its signal half-life is still only 5-10 minutes making it unsuitable for HTS applications. This decrease in activity, the result of slow degradation of the enzyme, leads to a lower rate of catalysis. This slower rate of catalysis corresponding results in a more stable luminescent signal. This is often accomplished by the addition of enzyme inhibitors. The Promega Steady-Glo kit uses a proprietary mix of enzyme inhibitors and buffer agents to obtain this stable signal. Note that the trade off with steady-state luciferase assays is sensitivity. HTS compatible steady-state “glow” assays are generally less sensitive than the equivalent “flash” assay [3].

Materials and Methods

Assay kits for Firefly luciferase enzyme activity quantification using glow (P/N E2510) and flash (P/N E1500) methodologies were purchased from Promega Corporation (Madison WI), as was purified recombinant firefly enzyme (Quantilum). All experiments used Corning Costar 3912 white opaque microplates.

Luciferase enzyme activity was measured using either a glow or a flash type assay following the suggested kit protocol. In each case, a series of dilutions of Quantilum recombinant firefly luciferase ranging from 0 to 10^{-12} moles/ml were made using 1X lysis buffer supplied in the luciferase kit. For glow type assays 10 µl each dilution were pipetted into microplates in replicates of 8, followed by 100 µl of Steady-Glo substrate, that had been mixed previously according to the kit instructions. After a 5-minute incubation at room temperature, the luminescence of the samples was measured using the Clarity™ Luminescence Microplate Reader (BioTek Instruments, Winooski, VT). Samples were measured kinetically for a 10 second interval, and recorded as an integral, as well as photons per second average. When the flash assay for luciferase was employed, 10 µl of each dilution were pipetted into microplates. The microplate was then read using the Clarity reader. During the read, 100 µl of luciferase substrate was added to each well and after a 2 second delay the luminescent signal was measured kinetically for a period of 10 seconds. The reader returned both an average signal per second as well as a total signal for the 10-second interval.

Results

These data presented demonstrate the Clarity reader’s ability to measure firefly luciferase levels using either flash or glow luminescent methodologies. The flash type kit requires the use of injectors to provide the necessary substrate prior to measuring the luminescence, while the steady state nature of the glow assay allows for the reagents to be added manually prior to luminescence measurements. Initial experiments compared the integrated luminescence data to the averaged photon per second data. When the two types of analysis were compared, no significant difference was observed in regards to the concentrations plots or the calculated detection limits (data not shown). Subsequent experiments used the averaged data.

The luminescence generated from firefly luciferase concentrations ranging from 1 x 10^{-22} to 1 x 10^{-14} moles per well was measured using a Clarity luminescence microplate reader (BioTek Instrument) and a Luciferase Assay System kit (Promega). When a concentration curve is plotted (Figure 2) an increasing relationship between concentration and luminescent output is observed. However the increase between 10^{-22} moles per well and 10^{-19} moles per well is substantially less than that observed from 10^{-19} to 10^{-14} moles per well (Figure 2). When the luminescence data from extremely low concentrations of enzyme are plotted a downward hook is seen at very low concentrations suggesting that some of the enzyme activity is being sequestered or inhibited. The most likely explanation is absorption to the sides of the microplate well. Using a signal to noise ratio test, the lowest enzyme concentration tested, 1 x 10^{-22} moles per well, was found to be significantly different than the buffer only blank wells.

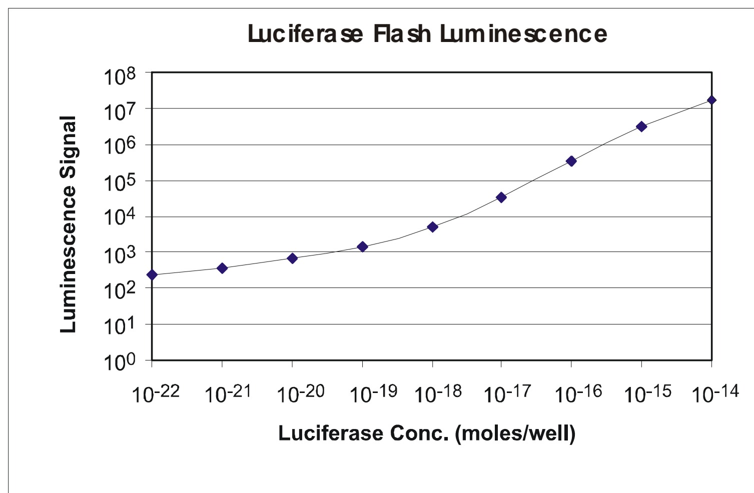

Figure 2. Firefly Luciferase Concentration Curve, Flash Luminescence. Various concentrations of firefly luciferase ranging from 10^{-14} to 10^{-22} moles per well were pipetted into microplates in replicates of 8 and the flash luminescence generated after the addition of Luciferin reagent was measured using a Clarity Luminescence Microplate reader (BioTek Instruments, Inc). The data was exported to Microsoft Excel® and plotted. Note that each data point (diamond) represents the mean of eight determinations.

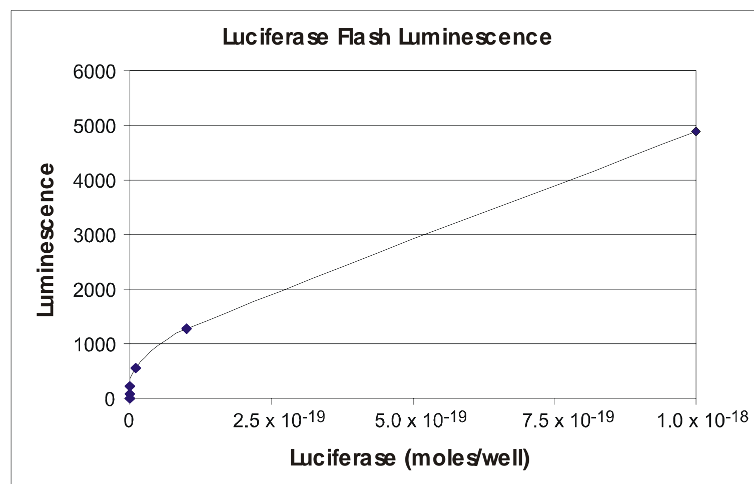

Figure 3. Flash Luminescence of Low Concentrations of Firefly Luciferase. Various concentrations of firefly luciferase ranging from 0 to 1 x10^{-18} moles per well were pipetted into microplates in replicates of 8 and the flash luminescence generated after the addition of Luciferin reagent was measured using a Clarity Luminescence Microplate reader (BioTek Instruments, Inc). The data was exported to Microsoft Excel® and plotted. Note that each data point (diamond) represents the mean of eight determinations.

The luminescence generated from similar firefly luciferase concentrations was also measured using a Clarity™ Luminescence Microplate Reader (BioTek Instrument) and a Steady-Glo kit (Promega). With this glow reaction, the luminescence response is quite minimal for concentrations below 10^{-19} moles per well (Figure 4). Once this threshold is reached the response is remarkably linear. As demonstrated in Figure 4, concentrations from 5 x 10^{-19} to 10^{-14} moles per well are quite linear. This is further corroborated by Figure 5, which is a similar concentration curve ranging from 2 x 10^{-19} to 1 x 10^{-16} moles per well. Linear regression analysis performed on these data show a very high correlation constant (r^2=1.00). A detection limit of 1 x 10^{-19} moles per well was calculated from these data when a signal to noise ratio test against the buffer only blank test was performed.

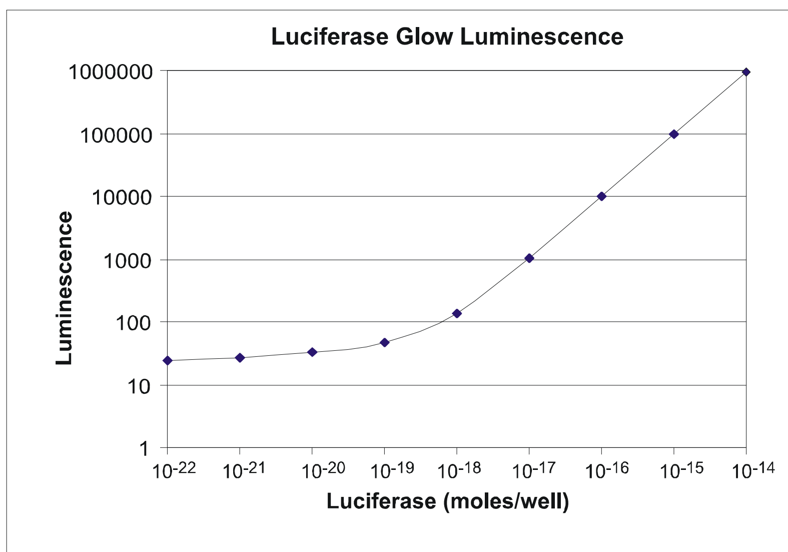

Figure 4. Firefly Luciferase Concentration Curve. Various concentrations of firefly luciferase ranging from 10^{-14} to 10^{-22} moles per well were pipetted into microplates in replicates of 8 and the glow luminescence was measured using a Clarity Luminescence Microplate reader (BioTek Instruments, Inc). The data was exported to Microsoft Excel® and plotted. Note that each data point (diamond) represents the mean of eight determinations.

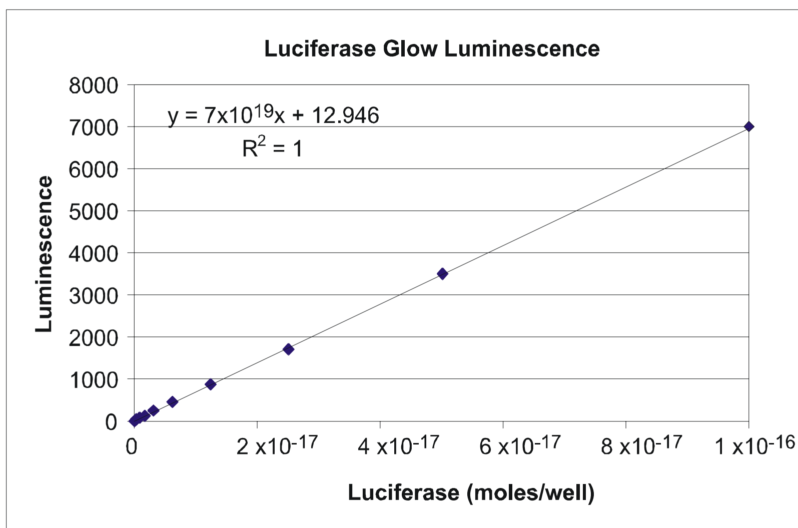

Figure 5. Glow Luminescence of Low Concentrations of Firefly Luciferase. Various concentrations of firefly luciferase ranging from 0 to 1 x10^{-16} moles per well were pipetted into microplates in replicates of 8 and the flash luminescence generated after the addition of Luciferin reagent was measured using a Clarity Luminescence Microplate reader (BioTek Instruments, Inc). The data was exported to Microsoft Excel® and plotted. Note that each data point (diamond) represents the mean of eight determinations.

Discussion

These data presented demonstrate the utility of the Clarity reader to perform luminescence determinations using luciferase enzyme as the reporter. Because luciferase enzyme is the most commonly used luminescent gene reporter it is paramount that a reader provide maximal sensitivity for this reaction. Using the more sensitive flash reaction the Clarity reader is capable of detecting as few as 60 protein molecules. Not surprisingly using the steady-state glow reactions results in less sensitivity. However, this is due to the chemistry of the reaction rather than any limitation of the reader.

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 Clarity reader can be configured in a number of different fashions depending on its intended use. For those users who only plan on performing glow or steady state assays, a model that does not have injectors is available. For flash type assays 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 (e.g. linear, orbital, and cross) are available for shaking of microplates. 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). 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.

References

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