ONE-Glo™ Luciferase Assay System: New Substrate, Better Reagent

ABSTRACT Early luciferase assay reagents required removal of assay components such as medium, serum and test compounds before measuring luminescence. The advent of homogeneous luciferase assays simplified reactions by eliminating the need for media removal prior to luminescence measurement and by enabling light measurement over an extended time period. Now a novel luciferin analog has been developed that enables luciferase assay reagents to operate at a lowered pH. This simple change leads to a reagent that is easier to use, more robust to the reaction environment and less aromatic than reagents containing unmodified luciferin.

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A novel luciferin substrate analog has been developed to improve luciferase reagent technology.

INTRODUCTION

Firefly luciferase assays have been used for more than 15 years to quantitate reporter gene expression. During this period, improvements in both luciferase genes and luciferase assay reagents have made luciferase assays one of the simplest reporter technologies available, both to implement and to read. Now a novel luciferin substrate analog has been developed. Based on this substrate, the ONE-Glo™ Luciferase Assay System[a,b,c] is a unique, homogeneous luciferase reagent that is more stable, more robust and less aromatic than standard luciferin reagents.

Luciferase enzyme expression is often quantified in studies ranging from signal transduction to protein:protein interactions, transcription and a wide assortment of other assay types (1,2). A monomer that requires no post-translational modification, firefly luciferase is well suited to these tasks. Furthermore, with the pGL4 Luciferase Reporter Vectors Promega improved the luciferase gene as a biological tool by optimizing mammalian expression, minimizing off-target responses and actively controlling protein half-life (3,4).

Quantifying luciferase in high-throughput and ultra-high-throughput environments requires a highly sensitive and robust reagent. Major improvements in luciferase reagent technology were made when homogeneous reagents were introduced. These early homogeneous reagents contained inhibitors to slow the luciferase reaction and thus prolong the luminescent signal. All subsequent reagents have been variations on this theme (5–7). These reagents work well but suffer limitations inherent in the luciferin chemical reaction and in the additives necessary to stabilize the luminescent signal.

We have created a new, proprietary reagent that contains a luciferin substrate analog, 5´-fluoroluciferin[a] (Figure 1). The addition of a fluorine at the 5´ position improves some of the fundamental characteristics of the luciferase reagent. Luciferase assays made with fluoroluciferin have lower thiol concentrations than conventional luciferase reagents and thus less thiol-associated odor. In addition, the fluoroluciferin-containing components and reconstituted reagents are much more functionally stable than similar luciferin-based reagents. Due to the presence of fluoroluciferin, the ONE-Glo™ Reagent is also more compatible with other compounds within the luciferase reaction, such as phenol red and small molecules that may inhibit luciferase.

IMPROVED COMPONENT AND REAGENT STABILITY

One of the unique characteristics of luciferase reactions containing fluoroluciferin is that the pH optimum for the reaction is much lower than for reactions containing conventional luciferin. Although the pH optimum of the luciferase-luciferin reaction is approximately 7.8 (8), pH 7.0 is optimal for the luciferase-fluoroluciferin reaction (Figure 2). In order to be useful as a substrate for luciferase, the 6´-OH of luciferin must be deprotonated. The fluorine in 5´-fluoroluciferin acts as an electron acceptor and lowers the pK of the 6´-OH to allow it to remain predominantly deprotonated at neutral pH.

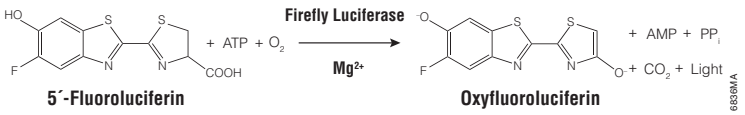

Figure 1. The luciferase reaction using 5´-fluoroluciferin.

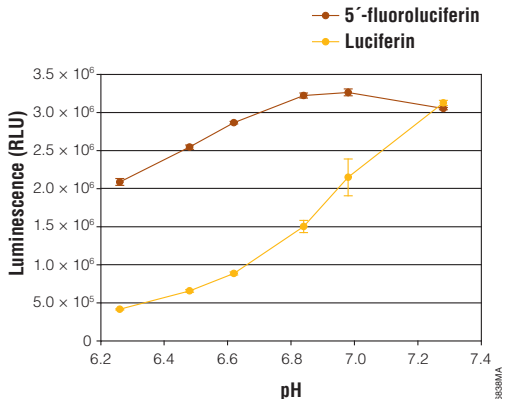

Figure 2. Luciferase reactions containing 5´-fluoroluciferin generate more light than those containing luciferin at low pH. The ONE-Glo™ Reagent formulation lacking 5´-fluoroluciferin was pH adjusted between 6.26 and 7.28. 5´-fluoroluciferin or luciferin was then added to the reagents. Reactions were initiated by adding an equal volume of DMEM containing 0.1% Prionex® (as carrier) and 14.9 ng/ml luciferase. Measurements were made 3 minutes after reaction initiation (n = 3).

The slightly acidic pH of fluoroluciferin-based reagents makes them more convenient to use, store, and reuse than conventional luciferases. The main cause of luciferase reagent inactivation is thought to be oxidation, and a lower pH protects the reconstituted reagent from oxidation. Luciferin-based reagents that generate high luminescence, like Bright-Glo™ Reagent or other bright-type reagents, decrease in functionality quickly, commonly by 10% every 4–5 hours at 22 °C. The reconstituted ONE-Glo™ Reagent decreases in functionality more slowly, 10% every 12–20 hours at 22 °C (Figure 3). Similar benefits are seen at 4 °C, where the ONE-Glo™ Reagent decreases in functionality by 12% over 5 days.

The lyophilized substrate component in the ONE-Glo™ System is similarly stabilized by the neutral to slightly acidic pH. Unlike most luciferin-based substrates, the lyophilized ONE-Glo™ Substrate can remain at room temperature for 3 weeks with only an 11% decrease in functionality. Alternatively, the substrate can be stored at 4 °C for several months before a measurable decrease in function occurs.

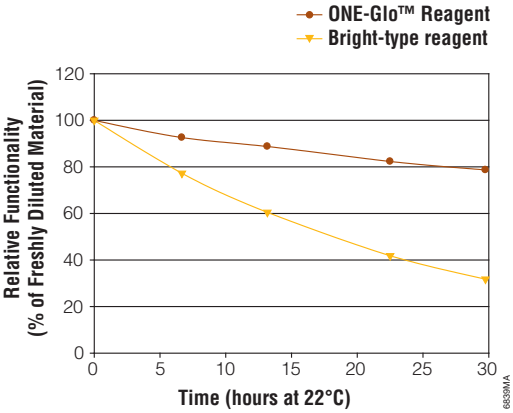

Figure 3. The reconstituted ONE-Glo™ Reagent can be used much longer than other commercially available bright-type reagents. Reconstituted reagents were placed at 22 °C and frozen at –70 °C at defined times. Upon thawing, the reagents were mixed with an equal volume of DMEM containing 14.9 ng/ml QuantiLum® Luciferase and 0.1% Prionex®. The relative activity was calculated as the luminescence intensity for each sample, measured 3 minutes after enzyme addition, relative to the intensity of the sample that was placed directly at –70 °C without 22 °C incubation (n = 3).

INCREASED TOLERANCE TO NONSUBSTRATE REACTION COMPONENTS

Fluoroluciferin and lowered pH make the ONE-Glo™ Reagent more tolerant to reaction components such as phenol red and small molecules that may inhibit luciferase.

The ONE-Glo™ Reagent was specifically designed to minimize the effects of nonessential compounds in the luciferase reaction environment. For example, phenol red is a common component in mammalian tissue culture medium (9). At the pH of typical luciferase reagents, phenol red absorbs a percentage of the emitted light. At lowered pH, the absorption spectrum of phenol red shifts, and the amount of color quenching is reduced. Hence, luminescence generated by the ONE-Glo™ Reagent is much less affected by compounds like phenol red.

This increase in reaction compatibility extends to compounds that decrease the luminescent signal by inhibiting the luciferase enzyme. Resveratrol is a well known inhibitor of firefly luciferase. Bakhtiarova *et al.* (10) observed roughly 80% inhibition of luciferase (20% of luciferase activity remained) in the presence of 10 µM resveratrol. The results of our studies with reactions containing 14.9 ng/ml firefly luciferase in DMEM + 0.1% Prionex® and 10 µM resveratrol are shown in Table 1.

Improvements in luciferase tolerance were also seen for inhibitors MPEP (Sigma Cat.# M5435) and SIB 1893 (Sigma Cat.# S9311) as shown in Table 1.

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Table 1. Retained Luminescence in the Presence of Various Luciferase Inhibitors.

Inhibitor	ONE-Glo™ Reagent	Bright-Glo™ Reagent	Bright-type Reagent
Resveratrol*	86 ± 2	71 ± 1	24 ± 2
MPEP	79 ± 1	—	34 ± 2
SIB1893	84 ± 1	—	46 ± 3

Retained luminescence is the amount of luminescent signal measured in the presence of inhibitor (= luminescence with inhibitor/amount of signal not inhibited × 100)
* n = 3

DECREASED THIOL, LESS ODOR

Luciferin-based reagents often contain inhibitors like AMP, phosphate and ammonium, or high concentrations of odorous thiols or reducing agents to slow luciferase turnover and generate a steady signal (5–7). These inhibitors can be greatly reduced in reactions that run at neutral or slightly acidic pH, however, because the lowered pH slows the enzyme turnover. Therefore, the use of fluoroluciferin generates bright luminescent signals and simplifies the composition of the reagent by removing the need for these inhibitors. Specifically, the ONE-Glo™ Reagent contains almost 100-fold less odor-causing thiols than the conventional homogeneous reagents. This also permits it to be shipped, used and disposed of without hazard warnings. Furthermore, the lack of these inhibitors also means that the luminescent signal generated by the ONE-Glo™ Reagent is both bright and stable when compared to Bright-Glo™ and Steady-Glo® Reagents (Figure 4).

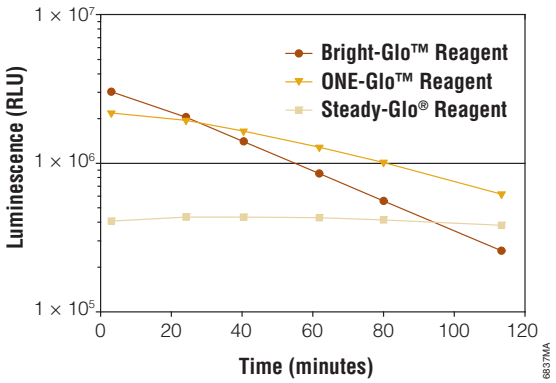

Figure 4. The ONE-Glo™ Reagent generates bright and stable luminescence that can easily be measured for multiple hours. Samples in 96-well plates consisted of 50 µl of purified firefly luciferase (14.9 ng/ml in DMEM with 0.1% Prionex®) combined with 50 µl of the respective reagent. Luminescence was measured (1.0 second integration/well) at 3 minutes and periodically for almost 2 hours. Coefficients of variation were < 3%; n = 3.

SUMMARY

With the ONE-Glo™ Reagent, Promega has developed an improved homogeneous luciferase reagent. While luciferase assays have traditionally had superior ease of use, incorporation of 5´-fluoroluciferin technology has enabled even further improvements to luciferase assay chemistry that are particularly useful when handling large quantities of samples, such as in a high-throughput screening environment.

REFERENCES

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PROTOCOL

- *ONE-Glo™ Luciferase Assay System Technical Manual*, #TM292, Promega Corporation
  www.promega.com/tbs/tm292/tm292.html

ORDERING INFORMATION

Product	Size	Cat.#
ONE-Glo™ Luciferase Assay System	10 ml	E6110
	100 ml	E6120
	1 L	E6130

For Laboratory Use.

(a)Patents Pending.

(b)The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

(c)Certain applications of this product may require licenses from others.

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