



# Steady-Glo™ Luciferase Assay System for High-Throughput Screening Applications

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*The Steady-Glo™ Luciferase Assay System<sup>(a,b)</sup> provides single-step quantitation of firefly luciferase expression in mammalian cell culture with extended-lifetime luminescence. The reagent is compatible with a broad range of culture media and is optimized for 96 and 384 well plates. Compared with conventional reagents having extended-lifetime luminescence, its unique formulation is more tolerant of mixing conditions, sample evaporation and pipetting errors. Thus, the Steady-Glo™ Reagent more readily supports good assay precision, particularly when used with automated sample handling instruments. These characteristics make the Steady-Glo™ Reagent particularly well suited for high-throughput batch analysis.*

## INTRODUCTION

The process of screening many thousands of samples to find only a few with the desired characteristics is the biochemical equivalent of "finding a needle in a haystack." A poorly designed screening method can result in little or no usable information, or so much statistical variability that valuable samples are obscured. The Steady-Glo™ Luciferase Assay System was developed to address these problems.

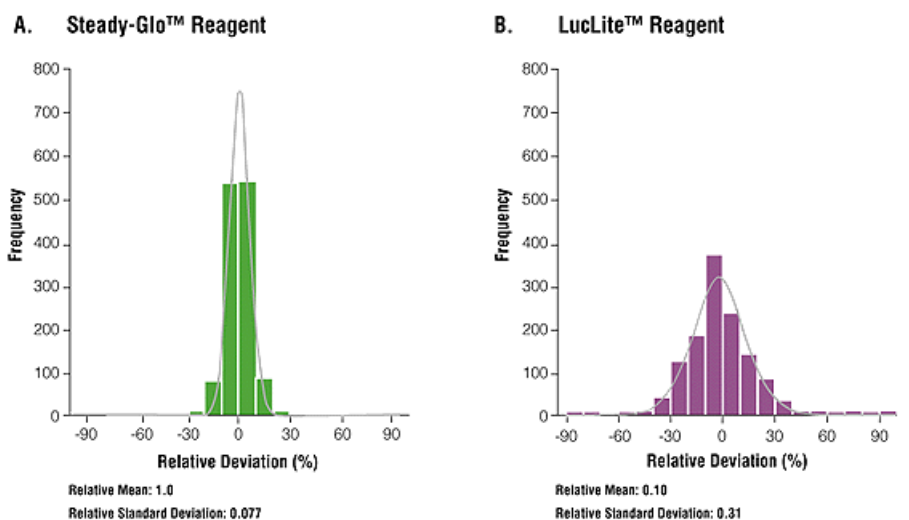
Firefly luciferase expression systems have become widely used for quantitative analysis of transcriptional modulation in living cells (1). In particular, this method enables libraries of small molecules to be rapidly screened for those affecting specific aspects of cellular physiology, such as receptor function or intracellular signal transduction. Other applications of firefly luciferase abound in academic and industrial settings.

However, the rapid collection of data is only a prerequisite to effective large-scale screening. The quality of data delivered by the screen is dependent upon assay precision. In automated screening of living cells, the sources of data error are usually attributed to the cell handling and automated processing procedures, while the role of reagent formulation in achieving reliable data is not adequately considered.

Batch processing of 96 or 384 well plates is the most common method for high-throughput screening of luciferase expression. Conventional reagents for this application were designed to provide extended luminescence in a homogeneous assay format, but with little consideration given to assay precision. In contrast, the novel formulation of the Steady-Glo™ Reagent not only provides extended luminescence, but does so without sacrificing fundamental principles of assay reliability. These principles are particularly important for high-throughput automated sample handling.

## PRECISION EXPRESSION SCREENING

The precision of the Steady-Glo™ Reagent was compared to that of a conventional extended-lifetime reagent using cells grown in 384 well plates (Figure 1). We used the LucLite™ Reagent (Packard Instruments) in this comparison, because it is widely used for this application; however, the principles described here are not unique to this particular reagent. Both systems require the addition of an equal volume of reagent to the culture medium in each well. The reagents induce cell lysis, and light emission results from the action of expressed luciferase on the luminescent substrates. In the example in Figure 1, 30µl of assay reagent were added to transgenic CHO cells grown in 30µl of F12 medium. The results show that the Steady-Glo™ Reagent consistently produced a smaller standard deviation than the LucLite™ Reagent. Although some of the data dispersion was undoubtedly due to variability in cell plating, the results show that reagent formulation can affect assay precision.



**Figure 1. Assay precision in 384 well plates.** The relative precision of luciferase measurements was determined using CHO cells stably transfected with the luciferase gene. High precision is indicated by a narrow distribution of measurements around the mean (solid lines show Gaussian distributions fitted to the data). In each of ten plates, half of the wells were measured using Steady-Glo™ Luciferase Assay System (**Panel A**) and the other half using LucLite™ Reagent (**Panel B**), each according to the manufacturer's instructions. For all samples, cells were grown in 30µl of F12 culture medium, to which an equal volume of assay reagent was added using a motorized pipettor. Luminescence was measured for 0.5 seconds per well using a Wallac 1450 MicroBeta® JET luminometer. The following variables had little effect on the data distributions: speed of reagent delivery by the pipettor, incubation interval after pipetting (1560 minutes), manufacturing lots of each reagent. Similar results were achieved using different cell lines. Note that these results are intended to show the relative effect of reagent formulation on assay precision; actual results may vary depending on equipment and experimental conditions.

To minimize effects of cell plating, all comparisons between the two reagents were made within the same plate. The reagents were added either on separate halves of the plate or in alternating rows. The results were examined for "edge effects" (e.g., variations caused by greater evaporation in outer wells). Assays performed on different days using different batches of cells yielded similar results, which could potentially bias the data. None was found in these experiments. Other potential sources of bias were examined by varying the amount of time between reagent addition and data collection (15 to 60 minutes), by changing the speed of reagent delivery, and by using two different manufacturing lots of both the Steady-Glo™ and LucLite™ Reagents. None of these factors significantly altered the results.

[Figure 1](#) is a composite of the experiments described above. Not only is the overall data dispersion of the LucLite™ Reagent about four-fold greater than that of the Steady-Glo™ Reagent, the distribution is also non-Gaussian. The number of data points occurring at more than two standard deviations from the mean is substantially greater than expected, which can present a problem when screening several thousand samples. In contrast, the distribution using the Steady-Glo™ Reagent is nearly Gaussian with very few extreme data points. In addition, the relative mean for the Steady-Glo™ Reagent is ten-fold greater than that of the LucLite™ Reagent. Under ideal conditions, the Steady-Glo™ Reagent generally yields 2.5- to 5-fold greater luminescence than the LucLite™ Reagent. This even larger difference in assay sensitivity is probably related to the difference in assay precision, as explained below.

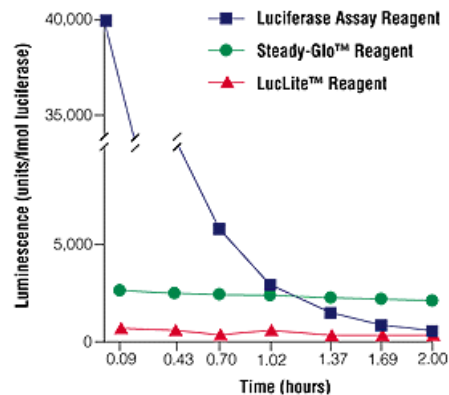
The difference in assay precision between the two reagent formulations remained essentially the same when tested using different cell lines and culture media. Even in an idealized simplification of a reporter assay, using purified firefly luciferase diluted into cell-free culture medium, the data dispersion of the Steady-Glo™ Reagent was about three-fold less than that of the LucLite™ Reagent. Although, for both reagents, the deviation from the mean was lower in the absence of cells.

## FORMULATED FOR MAXIMUM PRECISION

For general research applications, Promega previously introduced a unique luciferase assay reagent designed for maximum sensitivity and control of the assay environment. This reagent (Luciferase Assay System<sup>(b)</sup>; Cat.# E1500) differed from prior assay reagents by containing coenzyme A to provide a more stable luminescent signal (3,4). The luminescence is stable for more than one minute, having a half-life of approximately 15 minutes. However, this stability is insufficient for screening applications where several multiwell plates are processed as a single batch. Furthermore, the assay requires removal of the growth medium prior to cell lysis, which is generally not practical for batch processing.

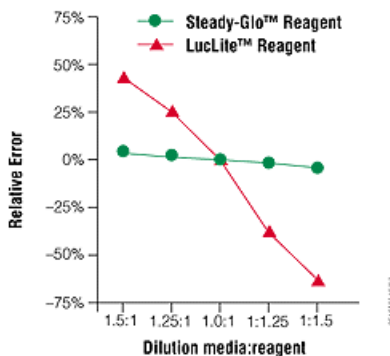
For applications requiring batch processing, a single-step homogeneous assay furnishing stable luminescence for an hour or more is preferred. However, luciferase reagents designed for maximum assay sensitivity cannot also provide sustained luminescence. Although the reasons for this are not fully understood, the luminescent signal slowly decays due to degradation of the enzyme during catalysis. To

reduce the rate of degradation, thereby providing more stable luminescence, the rate of catalysis must also be reduced (Figure 2). Hence, luciferase assays designed for extended luminescence have lower luminescence intensities, resulting in lower assay sensitivity. In large-scale screening applications, this trade-off in sensitivity is generally acceptable due to the premium placed on sample throughput.



**Figure 2. Signal kinetics for high sensitivity and extended-lifetime assay reagents.** Firefly luciferase was diluted to  $2.2 \times 10^{10}$ M in F12 medium containing 1mg/ml BSA and assayed using Promega's Luciferase Assay Reagent, Steady-Glo™ Luciferase Assay System and LucLite™ Reagent according to the manufacturer's instructions. The average of triplicate samples, each measured every 20 minutes over 2 hours, was recorded for each assay reagent. For each measurement, luminescence was integrated for 5 seconds using a Turner Designs Model 20E luminometer.

Lower catalytic rates can be achieved simply by adding an enzymatic inhibitor, however, the assay becomes susceptible to variations in the reagent concentration. Specifically, light output is overly inhibited if the reagent concentration is too high, and luminescence stability is reduced if the reagent concentration is too low. This sensitivity to reagent concentration is evident in conventional extended-lifetime assay reagents, such as the LucLite™ Reagent (Figure 3). This effect can reduce experimental precision under common laboratory conditions, particularly when automation is involved. The greatest concern is incomplete sample mixing, which yields non-homogeneous and variable reagent concentrations within each sample. Obtaining thorough mixing in high-throughput processing of 96 well plates can be difficult; it can be virtually impossible in 384 well plates due to the very small sample volumes. Reagent concentration can also vary as a result of sample evaporation or pipetting error.



**Figure 3. Effects of reagent concentration on assay precision.** Purified firefly luciferase ( $2.2 \times 10^{10}$ M in RPMI 1640 medium with 1mg/ml BSA) was added to a 96 well plate at 100µl per well. Either Steady-Glo™ Reagent or LucLite™ Reagent was then added to create the indicated dilutions. Luminescence measurements were integrated over 0.5 seconds per well using a Dynex MLX™ luminometer. Relative error was calculated as the percent change from the luminescence produced by the 1:1 dilution sample. The plotted data is the average of four wells (relative standard error <2.8%).

The formulation of the Steady-Glo™ Reagent largely overcomes this concentration effect (Figure 3). The luminescence intensity of the assay changes little over a wide range of reagent concentrations, making the reagent less sensitive to changes in sample volume, dispensing and mixing conditions. This allows good precision to be more easily achieved with automated liquid handling equipment, potentially reducing the occurrence of false positive or false negative results in high-throughput screening. In the experiments performed in Figure 1, the Steady-Glo™ Reagent consistently yielded substantially greater precision in 384 well plates under a variety of experimental conditions. The gain in precision was less pronounced in 96 well plates where sample mixing is more easily achieved, although the effect is still apparent.

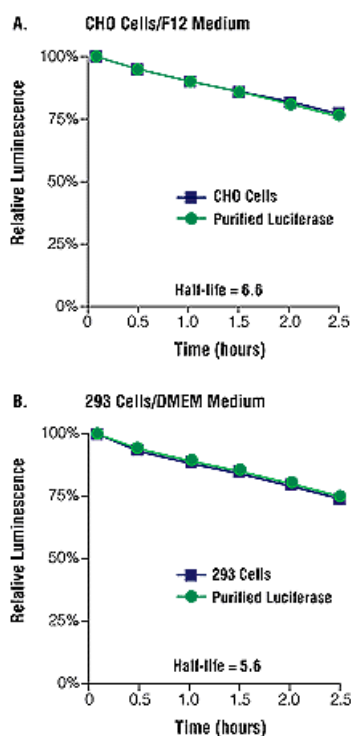
In principle, any reagent can provide high assay precision through rigorously controlled sample processing. However, such control is

difficult to achieve in practice without compromising sample throughput. The value of the Steady-Glo™ Reagent is its robustness to the moderate sample-to-sample variation typically incurred in high-throughput screening. For batch processing of multiwell plates, the Steady-Glo™ Reagent can provide reliable precision with less effort than conventional extended-lifetime reagents. In some cases, this assay robustness also further increases assay sensitivity. Although the Steady-Glo™ Reagent is about four-fold more sensitive than the LucLite™ Reagent in F12 culture medium under ideal conditions (Figure 2), for the data shown in Figure 1 it was consistently about ten-fold more sensitive. This increased sensitivity is likely due to overinhibition of the luminescence reaction caused by incomplete mixing of the LucLite™ Reagent in the small sample wells.

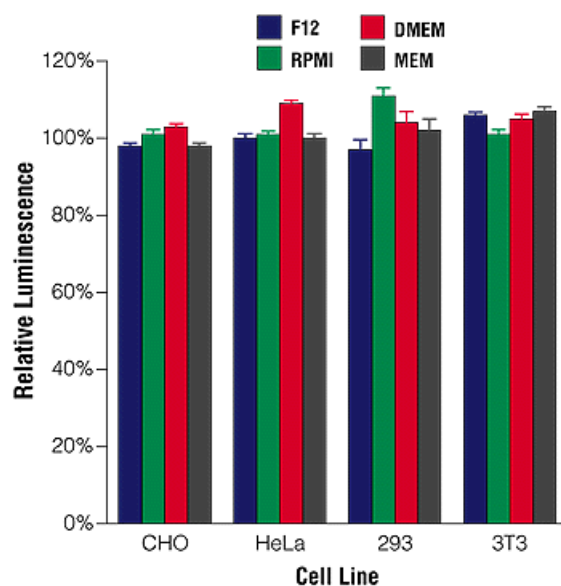
## OTHER ASSAY CHARACTERISTICS

The Steady-Glo™ Reagent provides a single-step luciferase assay that works directly in the cell culture medium without requiring prior sample processing. A luminescence signal having a half-life greater than five hours is achieved simply by adding the reagent to the cultured cells. The reagent is compatible with commonly used culture media for mammalian cells (RPMI 1640, MEMalpha, DMEM and F12), with or without added bovine or fetal calf serum. It is also tolerant of low concentrations of organic solvents (e.g., DMSO or isopropanol) or phenol red. (At higher concentrations of phenol red, the sensitivity of the assay is reduced somewhat due to light absorption by the indicator dye, but the luminescence stability is little affected.)

The ability to accurately quantitate luciferase expressed in living cells using the Steady-Glo™ Reagent can be demonstrated by comparing the luminescence with that of purified firefly luciferase. Ideally, the luciferase expressed by mammalian cells should produce a result equivalent to that of purified luciferase added to culture medium. Using cells stably transfected with the luciferase gene (either CHO cells in F12 medium or 293 cells in DMEM medium), the kinetics of the luminescence signal was virtually identical to that obtained with purified enzyme (Figure 4). While the medium composition affected the luminescence stability, the source of the luciferase or the presence of cells did not. In both cases, the luminescence half-life was greater than five hours. Quantitative release of luciferase from the cultured cells using the Steady-Glo™ Reagent was verified with a variety of cell types and media (Figure 5).



**Figure 4. Luminescence kinetics for purified firefly luciferase and luciferase expressed by stably transfected mammalian cells.** Samples in 96 well plates consisted of either 100  $\mu$ l of purified enzyme ( $2.2 \times 10^{12}$ M with 1mg/ml BSA) or mammalian cells ( $\sim 3 \times 10^5$  per well) that had been stably transfected with the luciferase reporter gene. **Panel A:** CHO cells in F12 medium. **Panel B:** 293 cells in DMEM. Luminescence measurements were integrated over 1 second per well using a Dynex MLX™ luminometer. As these data show, very little difference in relative luminescence is evident between the transfected cells and the purified enzyme in the same culture medium. The plotted data is the average of 48 wells (relative standard error <1%).



**Figure 5. Efficiency of cell lysis in four common cell lines.** The lysis efficiency of the Steady-Glo™ Luciferase Assay System was determined using four different cell lines, each stably transfected with the firefly luciferase gene. Luminescence from the cells was measured in 96 well plates using four different, commercially available culture media. Lysis efficiency was calculated as the luminescence measured after mixing 5 minutes with Steady-Glo™ Reagent divided by the luminescence of the same samples after one freeze-thaw cycle (80°C overnight). To correct for artifacts caused by the freezing and thawing on enzyme activity, the ratios were normalized to parallel samples of purified luciferase diluted in the same medium containing 1mg/ml BSA. Luminescence measurements were integrated over 1 second per well using a Dynex MLX™ luminometer. The plotted data are the averages of 16 wells containing cells and 8 wells containing purified enzyme.

In addition to the chemical properties described above, the physical properties of the Steady-Glo™ Reagent were also optimized for broad applicability and reliability of the assay. Temperature is a key physical property, since the luminescence is generated by an enzymatic reaction. The easiest means for maintaining a constant temperature is to perform all operations at room temperature, which is near the temperature optimum for firefly luciferase (2426°C). However, cold storage of the reagent substrates is required for maximum shelf life.

The Steady-Glo™ Reagent was designed to avoid the need to thaw or equilibrate the reagent before use. Only the reagent substrates require cold storage. These substrates are provided as a lyophilized solid, which dissolve almost immediately after the assay buffer is added. Since the buffer is provided in pre-measured aliquots and may be stored at room temperature, the reagent can be reconstituted within seconds without the need for lengthy temperature equilibration or volumetric measurements before use. In addition, reagent density, viscosity and foaming activity were optimized for maximum assay reproducibility and compatibility with standard laboratory equipment.

## CONCLUSIONS

Promega's Steady-Glo™ Luciferase Assay System has been developed specifically for high-throughput batch processing applications. The luminescent signal produced by the Steady-Glo™ Reagent has a half-life of more than five hours in common culture media, allowing multiple plates to be read in 1 to 2 hours with little change in luminescence. Since both cell lysis and luciferase activation are achieved by adding a single reagent directly into the cell culture medium, overall throughput is greatly increased. The Steady-Glo™ Reagent more readily supports high assay precision than conventional extended-lifetime luminescence reagents due to its tolerance of concentration differences caused by mixing conditions, sample evaporation and pipetting errors. The Steady-Glo™ Reagent's sensitivity and robustness result in high assay precision for high-throughput screening. In addition, assay precision is supported by relatively high assay sensitivity, robustness to cell culture conditions and quantitative cell lysis.

## REFERENCES

1. Wood, K.V. (1998) *Promega Notes* **65**, 14.
2. *Steady-Glo™ Luciferase Assay Reagent Technical Manual #TM051*, Promega Corporation.
3. Wood, K.V. (1991) In: *Bioluminescence and Chemiluminescence: Current Status*, eds. P. Stanley and L. Kricka, John Wiley and Sons, Chichester, 11.
4. *Luciferase Assay System Technical Bulletin #TB101*, Promega Corporation.

## Ordering Information

Product	Size	Cat.#
Steady-Glo™ Luciferase Assay System	10ml	<a href="#">E2510</a>
	100ml	<a href="#">E2520</a>

Please contact Promega for information on Bulk Purchases and Custom Orders.

<sup>(a)</sup>Patent Pending.

<sup>(b)</sup>U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289, and Australian Pat. No. 649289, have been issued to Promega Corporation for a firefly luciferase assay method, which affords greater light output with improved kinetics as compared to the conventional assay.

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