

Design of Homogeneous Luciferase Assays



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Homogeneous luciferase assays can provide quantitative analysis of luciferase expression with more speed and less effort than traditional luciferase assays. However, the design of homogeneous assays can substantially affect data quality and quantitative robustness. Of greatest concern is achieving uniform luminescence potential within the sample wells. This can be compromised by concentration-dependence of the assay reagent and by formation of luminescence gradients when the reagent is combined with cell culture media. The formulation of Promega's Steady-Glo™ Reagent^(a,b) contains innovative features that overcome these limitations.

INTRODUCTION

Homogeneous assays for firefly luciferase are becoming the standard means for rapid, precise analysis of gene regulation in mammalian cells. These assays yield a sensitive and quantifiable luminescent signal from the luciferase reporter upon the addition of a single reagent directly into the culture medium containing the cells. This is more convenient than traditional luciferase assays, which require removal of the culture medium and cell lysis prior to initiation of the luminescent reaction. By eliminating virtually all sample processing steps, homogeneous luciferase assays enable rapid analysis of many samples with little effort. The assays are particularly suited to analysis of cells grown directly in high-density multiwell plates, such as 96 well, 384 well or even 1,536 well plates. Thus, culture dishes containing the cells can be read directly in a luminometer without requiring sample transfer.

The more efficient assay method increases experimental confidence by allowing analysis of more sample replicates or incorporation of more control samples into the experimental design. Higher sample throughputs can also be achieved, making homogeneous luciferase assays the preferred choice for screening applications. A 96 well plate can be analyzed in 2–10 minutes with common laboratory equipment. Screens involving 10,000 to 100,000 luciferase assays per day are commonly performed in the pharmaceutical industry. In traditional luciferase assays, the luminescence signal is stable only for about one minute. The signal stability is extended in homogeneous assays to allow sufficient time for reagent addition to all sample wells prior to reading the plate. In high-throughput applications, signal stability is often greatly extended to enable processing of a stack of plates (typically 20–40 plates). In these extended-lifetime reagents, the luminescence signal slowly decays with a half-life usually greater than 5 hours.

Firefly luciferase assays have been widely used for more than a decade largely due to their quantitative robustness. Although the homogeneous luciferase assays can provide more data with less effort, they should be designed to maintain their high level of quantitative reliability. When quantifying gene expression using luciferase, it is generally appreciated that many factors can affect precision, such as how the cells are grown and handled or how effectively samples are processed by laboratory automation. However, the role of reagent formulation is generally not recognized as contributing significantly to assay precision. Previously we have shown that ineffective reagent design can increase experimental

error by several-fold (1). This can seriously hinder the advantages of homogeneous luciferase assays by compromising data integrity. It can be particularly troublesome in screening applications, where relatively rare events are sought from a very large number of samples. Poor assay precision results in statistical “noise,” which can obscure these events. This can lead to increased costs and greater risk of missed opportunities.

This article describes in more detail how assay precision and reliability can be affected by reagent formulation. We have found that precision can be substantially affected by a lack of uniform luminescence potential within sample wells. Normally, it is expected that a homogeneous luciferase reagent would uniformly support luminescence throughout the sample volume, but often this is not the case. Two conditions can cause nonuniformity: concentration-dependent luminescence caused by incomplete mixing, and formation of luminescence gradients after reagent addition. The effects of these conditions can be severe in some circumstances, causing large changes in luminescence output. Nonuniformity due to reagent formulation may sometimes be the major contributor to experimental error. Fortunately, these conditions can be minimized through proper reagent design, thus providing homogeneous assays with the quantitative robustness of traditional luciferase assays.

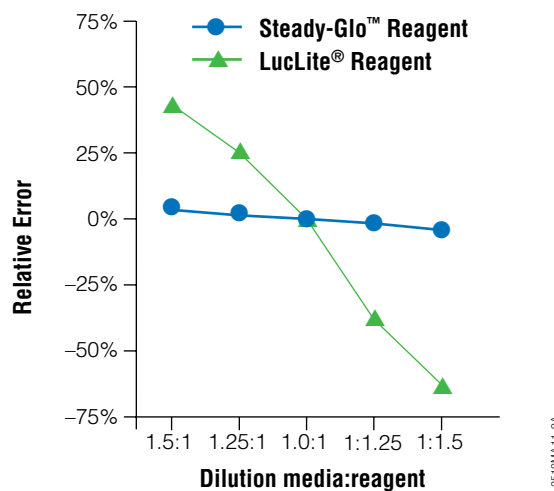


Figure 1. Concentration-dependent luminescence. Purified firefly luciferase (2.2×10^{-10} M in RPMI 1640 medium with 1mg/ml BSA) was added to a 96 well plate at 100 μ l per well. Either Steady-Glo™ Reagent or LucLite® Reagent was then added to create the indicated dilutions. Luminescence measurements 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%). A systematic change in relative error vs. dilution indicates concentration dependence in reagent performance.

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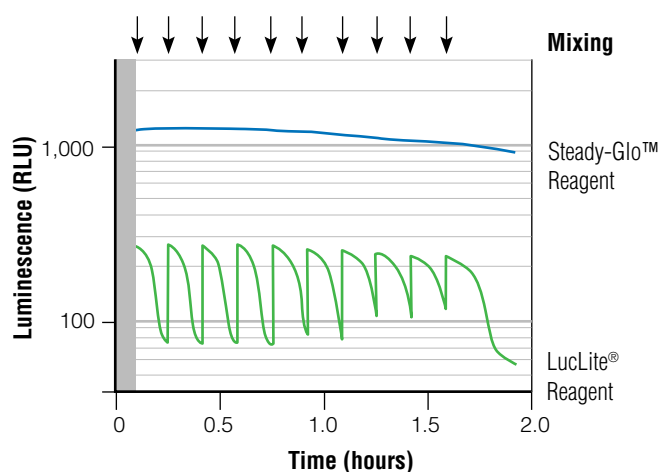


Figure 2. Effect of luminescence gradients on sample mixing. Luminescent reactions were initiated by adding 100 μ l of assay reagent to 100 μ l of purified luciferase in F12 medium (2.4×10^{-10} M luciferase with 1mg/ml BSA). Reactions were performed in 8 \times 50mm tubes, which have similar dimensions to single wells of a 96 well plate. Luminescence from the tubes was read continuously in a Turner Designs Model 20e luminometer. At 10-minute intervals, the tubes were removed from the luminometer, briefly mixed by vortexing and returned to the luminometer. The decrease in luminescence following mixing is caused by depletion of oxygen by the assay reagent. Mixing the tubes re-introduces oxygen, causing the luminescence to return to its initial intensity. The Steady-Glo™ Reagent was designed to minimize the formation of luminescence gradients. Similar results were achieved when samples containing mammalian cells expressing luciferase were used.

CONCENTRATION-DEPENDENT LUMINESCENCE

Concentration-dependent luminescence can arise when homogeneous reagents are designed for extended-lifetime luminescence. Through an unknown mechanism, the catalysis of light production by luciferase also leads to inactivation of the enzyme. This can be minimized through proper reagent formulation, but it cannot be completely overcome. Reagents designed to maximize luminescence output, and thereby provide maximum assay sensitivity, have relatively short signal duration. For example, Promega's Luciferase Assay System^(b) has a half-life of about 15 minutes. To provide assay reagents with extended-lifetime luminescence, the rate of inactivation must be further reduced by also reducing the rate of catalysis. Hence, reagents with extended-lifetime luminescence also have reduced assay sensitivity.

The reduction in sensitivity is dependent on the extent of signal duration and on the effectiveness of other formulation strategies to protect against inactivation. Promega's Steady-Glo™ Reagent is a homogeneous luciferase reagent designed to provide a luminescence half-life greater than 5 hours. Although the reagent is designed for optimum luminescence output, with extended duration of the luminescence signal, the assay sensitivity is reduced about 20-fold. The assay characteristics are dependent in part on medium composition since homogeneous assays are performed in cell culture medium. Thus, assay sensitivity can differ somewhat depending on the type of culture medium used. Nevertheless, the reduction in assay sensitivity is generally not a limitation, and the trade-off for greater luminescence duration makes the Steady-Glo™ Reagent the preferred choice for many applications.

To extend luminescence duration by reducing catalytic rate, the obvious strategy is to add an inhibitor to the reagent formulation. Unfortunately, this can also make reagent performance strongly concentration-dependent since enzymatic turnover is controlled by the amount of inhibitor present. This effect can be seen in the LucLite® Reagent (Packard Instruments). Concentration-dependence can be made apparent by

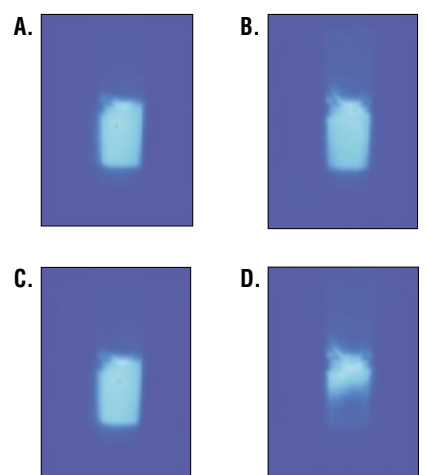


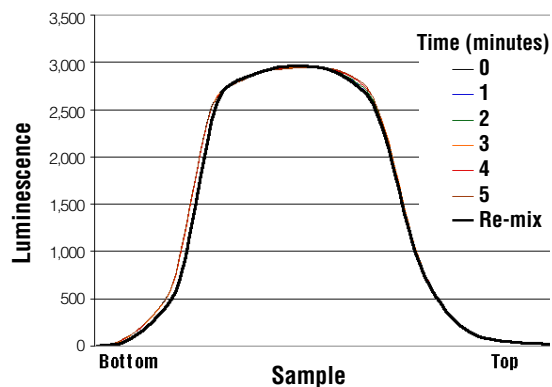
Figure 3. Luminescence gradients. The formation of luminescence gradients can be visualized in the wells of a 384 well plate using the NightOwl® CCD-imaging camera (EG&G Berthold). The luminescence reactions were initiated by adding 20 μ l of assay reagent to 20 μ l of purified luciferase in F12 medium (2.2×10^{-8} M luciferase with 1mg/ml BSA). Both the Steady-Glo™ Reagent (Panel A) and the LucLite® Reagent (Panel B) show uniform luminescence immediately after mixing the sample. After 12 minutes, the luminescence from the Steady-Glo™ Reagent remains uniform (Panel C), but the luminescence from the LucLite® Reagent (Panel D) has faded at the bottom of the well. For clarity, the relative image intensity of each panel has been adjusted (see Figure 4).

adjusting the ratio of reagent to culture medium and measuring luminescence output (Figure 1). Both Steady-Glo™ Reagent and LucLite® Reagent are designed to be mixed in equal volume with the culture medium (i.e., 1:1 ratio). If the LucLite® Reagent is too concentrated (i.e., ratios of 1:1.25 and 1:1.5), too much inhibitor is present, causing luminescence to decrease. Conversely, if the LucLite® Reagent is too dilute (i.e., ratios of 1.5:1 and 1.25:1), luminescence increases, causing a concomitant decrease in signal duration. The Steady-Glo™ Reagent employs a different strategy to overcome this problem. Luminescence output and duration change little with differing concentrations of Steady-Glo™ Reagent.

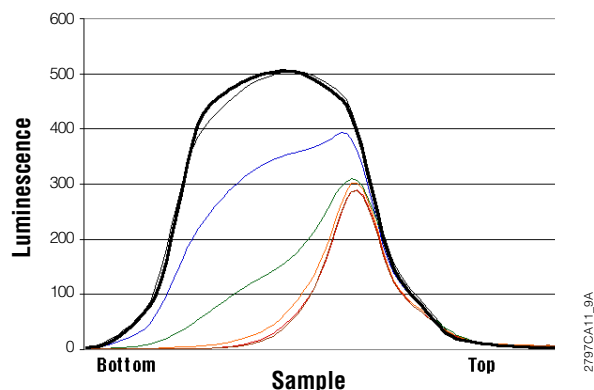
The importance of concentration-dependence will depend on how samples are handled. Under circumstances where assay reagent is accurately pipetted and mixed thoroughly in each sample, the effect of concentration-dependence will be negligible. This is typically the case when a relatively small number of samples are processed manually. However, concentration-dependence becomes more important in automated systems. In high-throughput operations involving large numbers of samples, it becomes more difficult to achieve consistently accurate pipetting and sample mixing. Mixing is usually accomplished simply by the action of the automated pipettor. Thus, although the correct volume of reagent may be added to each sample, without complete mixing, some portions of the sample well will be too concentrated and some portions too dilute. Because Steady-Glo™ Reagent shows little concentration dependence, nonuniformity due to incomplete mixing is not a problem. Even though the reagent concentration may vary within sample wells, reagent activity remains virtually unaffected. This is particularly important for very small sample wells where mixing is more difficult (e.g., in 384 well plates) or when the sample itself is nonuniform (e.g., attached cells).



A. Steady-Glo™ Reagent



B. LucLite® Reagent



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Figure 4. Formation of luminescence gradients. The formation of luminescence gradients can be quantified approximately using the NightOwl® CCD-imaging camera. To avoid optical aberrations due to the plastic of multiwell plates, the luminescent reactions were measured in glass cuvettes (2mm width; 1cm length). The luminescence reactions were initiated by adding 50µl of assay reagent to 50µl of purified luciferase in F12 medium (2.2×10^{-8} M luciferase with 1mg/ml BSA). The rate of formation of the luminescence gradient was greater than that in 384 well plates due to the sample volume constraint imposed by the cuvette. **Panel A.** Steady-Glo™ Reagent. **Panel B.** LucLite® Reagent. The results show how the luminescence of the LucLite® Reagent fades from the bottom of the sample, and also diminishes from the top of the sample. After remixing the sample, uniform luminescence returns throughout the sample volume. In contrast, the luminescence of the Steady-Glo™ Reagent remains uniform throughout the sample.

LUMINESCENT GRADIENTS

Luminescent gradients can form in samples despite complete mixing. Although the phenomenon is not completely understood, gradients apparently form due to the availability of oxygen to the luminescent reaction. Empirically it is observed that uniform luminescence is initially achieved throughout the sample volume upon mixing luciferase with a homogeneous assay reagent. However, within minutes luminescence can begin to fade from the bottom of the sample well, leaving a bright reaction only at the top. Upon mixing, the reaction will return to uniform luminescence to begin fading again (Figure 2). These gradients can cause the luminescence output of a luciferase reaction to diminish significantly. Furthermore, because these gradients cannot easily be prevented by sample handling techniques, they pose a potentially greater hindrance to assay precision.

The gradients are probably caused by chemical reaction of dissolved oxygen with the strongly reducing environment of the luciferase reagent. Luciferase reagents require a reducing environment to sustain bright luminescence. However, within minutes the reducing environment can deplete the oxygen needed for the luminescent reaction. When this happens, luminescence is supported only at the top of the reaction vessel, where oxygen can diffuse into solution. What is not understood is the role of the culture medium in which the homogeneous assay is performed. The depletion of oxygen is very slow if buffered saline is used in place of culture medium. Furthermore, different types of media affect the rate of oxygen depletion. Oxygen depletion is greatest with F12 or MEM α media. The effect can also differ substantially with different manufacturers of the same media or with different lots from the same manufacturer. As expected, the gradients will also be affected by oxygen diffusion due to the geometry of the sample wells. The gradients will form more rapidly in tall and narrow sample wells, such as found in 384 well plates, compared to shallow sample wells.

Luminescence gradients can be clearly seen when the LucLite® Reagent is added to purified luciferase in F12 medium (Figure 3); within 10 minutes, the luminescence from the bottom of the sample wells is virtually extinguished (Figure 3, Panel D). Luminescence remains at the top of the sample, but even that is limited by the rate of oxygen diffusion (Figure 4, Panel B). Upon remixing, luminescence is returned throughout the sample well. In contrast, luminescence from the Steady-Glo™ Reagent remains uniform throughout the sample well under the same conditions (Figure 3, Panel C, and Figure 4, Panel A). The Steady-Glo™ Reagent is formulated to minimize oxygen consumption by the reagent, resulting in negligible loss of luminescence over time. It should be noted that oxygen depletion is not caused by oxygen consumption in the bioluminescence reaction. For example, luminescence gradients can form rapidly in the LucLite® Reagent even although the luminescence intensity is less than the Steady-Glo™ Reagent.

The formation of luminescence gradients reduces assay precision by establishing an unstable chemical environment. In particular, any vibration or inadvertent agitation of samples containing luminescence gradients will cause the luminescence output to change. Such physical agitation is difficult to avoid while handling samples either manually or with automated instrumentation. Furthermore, even without physical agitation, the ability to form a reproducible balance between oxygen diffusion and depletion is tenuous. The balance will be affected by slight changes in the sample meniscus, such as differences in the wetting of the sample wells, occurrence of air bubbles, or changes in air flow around the wells. In addition, formation of the luminescence gradient reduces luminescence output and thus assay sensitivity. Although using buffered saline for all measurements could minimize the effect, it would defeat the purpose of using a homogeneous reagent as it would require removal of the culture medium prior to measurement. It is preferable to use a reagent designed to overcome this effect and thus avoid the problem altogether.



CONCLUSION

The purpose of homogeneous luciferase assays is to provide rapid and quantitative analysis of gene expression. These characteristics should not be compromised by limitations of reagent formulation. We have found that assay precision can be substantially hindered by nonuniformity of luminescence potential within sample wells. This can be caused by concentration-dependence of the assay reagent and by formation of luminescence gradients after the reagent is combined with culture media. By reducing assay precision, these conditions reduce the quantitative robustness and potentially the data integrity of luciferase measurements. More fastidious sample processing can compensate for these deficiencies, but generally at the cost of assay convenience and throughput. Fortunately, as we have shown for the Steady-Glo™ Reagent, these problems can be overcome through careful design of the reagent formulation.

The Steady-Glo™ Reagent is more tolerant of variations in reagent concentration, allowing it to be more forgiving of pipetting errors and incomplete sample mixing. These characteristics make the Steady-Glo™ Reagent particularly suited for use with automated systems, such as those commonly used in screening applications. The Steady-Glo™ Reagent also maintains stable, uniform luminescence in cell culture medium, providing greater sensitivity and quantitative robustness in homogeneous assays. These combined features make the Steady-Glo™ Reagent a simpler and faster alternative to traditional luciferase assays, without sacrificing data quality. The Steady-Glo™ Reagent has also been formatted to allow easier storage and preparation than any other luciferase assay. The reagent can be prepared for use in seconds without measuring, thawing or temperature pre-equilibration.

Because the LucLite® Reagent is a common and familiar homogeneous luciferase reagent, it was used to illustrate the effects of reagent formulation on assay performance. However, the assay characteristics described here are not unique to the LucLite® Reagent. It is hoped that by describing the underlying causes that limit assay performance, it is apparent that these problems are generic to the basic design of homogeneous luciferase assays. These problems are common to other commercial and noncommercial reagent formulations. The Steady-Glo™ Reagent contains several innovative features to overcome these limitations, thus providing high-quality reagent performance for consistently rapid and quantitative luciferase measurements.

REFERENCE

1. Hawkins, E., Jennens-Clough, M. and Wood, K.V. (1999) *Promega Notes* 70, 7.



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Ordering Information

Product	Size	Cat.#
Steady-Glo™ Luciferase Assay System	10ml	E2510
	100ml	E2520
	10 × 100ml	E2550

Steady-Glo is a trademark of Promega Corporation.

LucLite is a registered trademark of Packard Instrument Company, Inc. *MLX* is a trademark of Dynex Technologies, Inc. *NightOwl* is a registered trademark of EG&G Berthold.

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