

Steady-Glo[®] Luciferase Assay System

INSTRUCTIONS FOR USE OF PRODUCTS E2510, E2520 AND E2550.

Steady-Glo[®] Luciferase Assay System

All technical literature is available on the Internet at: www.promega.com/tbs/
Please visit the web site to verify that you are using the most current version of this Technical Manual. Please contact Promega Technical Services if you have questions on use of this system. E-mail: techserv@promega.com

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1. Description

High-throughput quantitation of luciferase expression in mammalian cells is commonly performed by batch processing of 96- and 384-well plates. The Steady-Glo[®] Luciferase Assay System^(a,b,c) is designed for this purpose, providing long-lived luminescence when added to cultured cells. The homogeneous assay provides a signal half-life of more than 5 hours in commonly used cell culture media without prior sample processing. Throughput rates of several thousand samples per hour may be achieved with high reproducibility under standard laboratory conditions.

The provided Steady-Glo[®] Luciferase Assay Buffer and Substrate are combined (see Section 3.B) to form Steady-Glo[®] Reagent.

Selected Citations Using the Steady-Glo[®] Luciferase Assay System:

- Rose, S.D. *et al.* (2005) Functional polarity is introduced by Dicer processing of short substrate RNAs. *Nucleic Acids Res.* **33**, 4140-56.

Having observed that blunt 27mers had increased potency in RNAi compared to 21mers or 27mers with 3' or 5' overhangs, the authors investigated the differences that may account for these changes in gene silencing activity using the same target sequence in enhanced green fluorescent protein (EGFP). In an EGFP RNAi experiment, the Steady-Glo[®] Luciferase Assay System was used to monitor firefly luciferase activity to normalize transfection of HEK 293 cells. A further RNAi experiment targeted the firefly luciferase gene in the pGL3-Control Vector cotransfected with 20, 2 or 0.4nM siRNA duplexes into HeLa cells. After 48 hours, the cells were lysed and assayed using the Luciferase Assay System.

- Kurata, S. *et al.* (2004) p51/p63 controls subunit 3 of the major epidermis integrin anchoring the stem cells to the niche. *J. Cell Biol.* **279**, 50069-77.

HeLa and Saos-2 cells were transiently transfected with luciferase reporter vectors made from the pGL3-Promoter Vector containing various intron sequences from the human integrin $\alpha 3$ gene IIGA3. The first intron sequence was originally cloned into the pGEM[®]-T Easy Vector. Cell cultures were assessed for luciferase activity by making lysates with the Glo Lysis Buffer and assaying with the Steady-Glo[®] Assay System.

2. Product Components and Storage Conditions

Product	Size	Cat. #
Steady-Glo [®] Luciferase Assay System	10ml	E2510

Each system contains sufficient reagents for 100 assays of 100 μ l each. Includes:

- 10ml Steady-Glo[®] Luciferase Assay Buffer
- 1 vial Steady-Glo[®] Luciferase Assay Substrate (lyophilized)
- 1 Protocol

Product	Size	Cat. #
Steady-Glo [®] Luciferase Assay System	100ml	E2520

Each system contains sufficient reagents for 1,000 assays of 100 μ l each. Includes:

- 100ml Steady-Glo[®] Luciferase Assay Buffer
- 1 vial Steady-Glo[®] Luciferase Assay Substrate (lyophilized)
- 1 Protocol

Product	Size	Cat. #
Steady-Glo [®] Luciferase Assay System	10 \times 100ml	E2550

Each system contains sufficient reagents for 100 assays of 100 μ l each. Includes:

- 10 \times 100ml Steady-Glo[®] Luciferase Assay Buffer
- 10 vials Steady-Glo[®] Luciferase Assay Substrate (lyophilized)

Storage Conditions: Store the lyophilized Steady-Glo[®] Substrate at -20°C. The substrate also may be stored at 4°C for up to one month. Store the Steady-Glo[®] Buffer below 25°C. Storage at room temperature is recommended to prevent the need for temperature equilibration when the reagent is reconstituted. Use the reconstituted reagent on the same day it is prepared, or store at -20°C for up to two weeks.

Note: Cat.# E2550 is provided in one box containing two trays: a 10-pack tray of Steady-Glo[®] Substrate to be stored at -20°C, and a 10-pack tray of Steady-Glo[®] Buffer to be stored unfrozen but below 25°C.

CAUTION: The lyophilized Steady-Glo[®] Substrate contains dithiothreitol (DTT) and is therefore classified as hazardous. The reconstituted reagent is not known to present any hazards, as the concentration of DTT is less than 1%. However, we recommend the use of gloves, lab coats and eye protection when working with these or any chemical reagents. Promega assumes no liability for damage resulting from handling or contact with these products.

Steady-Glo® Luciferase Assay System Protocol

3. Performing the Steady-Glo® Luciferase Assay


3.A. General Considerations

The Steady-Glo® Luciferase Assay System is designed for use with the following culture media containing 0–10% serum: RPMI 1640, MEM α , DMEM and Ham's F12. The signal half-life under these conditions is greater than 5 hours at 22°C (less than 13% loss of luminescence per hour) and is independent of enzyme concentration where the luciferase concentration is $<10^{-8}$ M. Other media/sera combinations also can be used, but experimental verification of assay performance is recommended in these cases (see Section 5.B). The luminescence signal also can be affected by changes in temperature or the presence of phenol red and organic solvents (see Section 5.B).

 Additional information on the Steady-Glo® Luciferase Assay Reagent can be found in Section 5.

Because the luminescent signal is affected by assay conditions, results should be compared only between samples measured using the same medium/serum combination. For analysis of multiple plates, greatest accuracy can be obtained by including a common control sample in each plate. By this method, luminescence measurements of each plate can be normalized to the control contained within the same plate. This allows the correction of small variations in luminescence that can occur over time or due to other variables such as temperature.

The Steady-Glo® Reagent should be added to plates at least 5 minutes prior to quantifying luminescence, as several minutes are required for cell lysis.

 The Steady-Glo® Luciferase Assay Reagent is **not** designed for use with automated reagent injectors that are integrated into some luminometers.

To achieve linear assay performance at low light levels, background luminescence must be subtracted from all readings. No background is produced by the Steady-Glo® Reagent or by mammalian cells lacking the luciferase gene, so background luminescence is a characteristic of luminometer performance. Some instruments also require verification of linear response at high light levels (consult the instrument manual).

Approximate stability of Steady-Glo® Reagent after reconstitution: 7% loss of luminescence per 8 hours at room temperature, 10% loss per 24 hours at 4°C and 8% loss per 2 weeks at –20°C. The reagent may be subjected to up to five freeze-thaw cycles with no effect on potency.

Steady-Glo® Luciferase Assay System Protocol

3.B. Reagent Preparation

To prepare the Steady-Glo® Reagent, transfer the contents of one bottle of Steady-Glo® Buffer to one bottle of Steady-Glo® Substrate. Mix by inversion until the substrate is thoroughly dissolved.

Notes:

1. Since luciferase activity is temperature-dependent, the temperature of the Steady-Glo® Reagent should be held constant while quantitating luminescence. This is achieved most easily by using Steady-Glo® Reagent equilibrated to room temperature, which is near the temperature optimum of luciferase (Section 5.B). Equilibration of the reagent prior to use is unnecessary when the Steady-Glo® Buffer is stored at room temperature.
2. Reagent stored frozen after reconstitution should be thawed below 25°C to ensure reagent performance. Mix well after thawing. The most convenient and effective method to thaw or temperature equilibrate cold reagent is to place it in a water bath at room temperature.
3. For maximum reproducibility, equilibrate cultured cells to room temperature before adding the Steady-Glo® Reagent.
4. Thorough sample mixing is required for maximum assay reproducibility, even though the effect of incomplete mixing is minimized by the formulation of the Steady-Glo® Reagent (Section 5.A). The characteristics of some automated pipettors may allow sufficient mixing upon reagent delivery, but this should be verified for individual circumstances (Section 5.B).

3.C. Assay Procedure

1. Remove 96- or 384-well plates containing mammalian cells from the incubator. The plates must be compatible with luminescence measurements in the luminometer being used.
2. Add a volume of reagent equal to that of the culture medium in each well and mix. For 96-well plates, typically 100 μ l of reagent is added to cells grown in 100 μ l of medium.
3. Wait a minimum of 5 minutes to allow sufficient cell lysis, then measure luminescence with a luminometer (consult instrument manual).

Note: Glo Lysis Buffer has been developed by Promega for use in nonhomogeneous assays with the Steady-Glo® Assay System. Glo Lysis Buffer protocol information can be found in Section 5.C.

4. Related Products

Luciferase Assay Systems

Product	Size	Cat. #
Bright-Glo™ Luciferase Assay System	10ml	E2610
	100ml	E2620
	10 × 100ml	E2650
Dual-Glo™ Luciferase Assay System	10ml	E2920
	100ml	E2940
	10 × 100ml	E2980
Dual-Luciferase® Reporter Assay System	100 assays	E1910
Dual-Luciferase® Reporter Assay System 10-Pack	1,000 assays	E1960
Dual-Luciferase® Reporter 1000 Assay System	1,000 assays	E1980
Luciferase Assay System	100 assays	E1500
Luciferase Assay System with Reporter Lysis Buffer	100 assays	E4030
Luciferase Assay Reagent, 10-Pack	1,000 assays	E1501
Luciferase Assay System Freezer Pack	1,000 assays	E4530
Luciferase 1000 Assay System	1,000 assays	E4550
Luciferase Assay Reagent	1,000 assays	E1483

Firefly Luciferase Reporter Vectors

Product	Size	Cat.#
pGL4.10[<i>luc2</i>] Vector	20µg	E6651
pGL4.11[<i>luc2P</i>] Vector	20µg	E6661
pGL4.12[<i>luc2CP</i>] Vector	20µg	E6671
pGL4.13[<i>luc2/SV40</i>] Vector	20µg	E6681

Additional firefly luciferase vectors at: www.promega.com/vectors/

Miscellaneous Luciferase Products

Product	Size	Cat.#
QuantiLum® Recombinant Luciferase	1mg	E1701
	5mg	E1702
Glo Lysis Buffer	100ml	E2661

Plasmid DNA Purification Systems

Product	Size	Cat.#
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495
PureYield™ Plasmid Maxiprep System	2 preps	A2391
	10 preps	A2392
	25 preps	A2393

5. Appendix

5.A. Overview of the Steady-Glo® Luciferase Assay System

Transcriptional regulation, coupled to the expression of a reporter gene, is routinely used to study a wide range of physiological events. A common example is the analysis of receptor function by quantifying the action of specific receptor response elements on gene expression. Other examples include the study of signal transduction, transcription factors, protein:protein interactions, and viral infection and propagation (1,2). Events downstream of transcription, such as mRNA processing and protein folding, also can be analyzed.

Luciferase is a popular choice as a reporter for these applications because functional enzyme is created immediately upon translation, and the assay is rapid, reliable and easy to perform (3,4). Furthermore, analysis using luciferase as the genetic reporter is well suited to laboratory automation and high-throughput applications. For these reasons, luciferase is widely used in the biotechnology and pharmaceutical industries.

Firefly Luciferase

Firefly luciferase is a 61kDa monomer that catalyzes the mono-oxygenation of beetle luciferin (Figure 1). Beetle luciferin is a relatively stable molecule found only in luminous beetles (which include fireflies). The enzyme uses ATP as a cofactor, although most of the energy for photon production comes from molecular oxygen. The quantum yield is about 0.9, the highest of any known luminescent reaction (5). The gene encoding firefly luciferase (*luc*) is a cDNA clone that has been incorporated into a number of reporter vectors (Section 4).

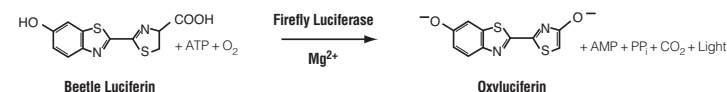


Figure 1. The luciferase reaction. Mono-oxygenation of luciferin is catalyzed by luciferase in the presence of Mg²⁺, ATP and molecular oxygen.

5.A. Overview of the Steady-Glo® Luciferase Assay System (continued)

Development of the Assay for High-Throughput Applications

For general research applications, Promega previously introduced a unique luciferase assay reagent designed for maximum sensitivity and control of the assay environment (6). This reagent differed from prior assay reagents by containing coenzyme A to provide a more stable luminescent signal (Luciferase Assay System, Cat.# E1500). However, for high-throughput applications where several multiwell plates are processed as a single batch, the signal stability of this reagent is insufficient, having a half-life of approximately 5–10 minutes. Furthermore, maximum control of the assay environment requires removal of growth medium prior to cell lysis, which is generally not practical for batch processing.

The Steady-Glo® Luciferase Assay System was developed specifically for batch processing of multiple plates in high-throughput applications. The luminescent signal produced by the Steady-Glo® Reagent has a half-life of more than 5 hours in common culture medium, allowing multiple plates to be read in 1–2 hours with little change in luminescence. Since both cell lysis and luciferase activation are achieved by adding a single reagent directly to the cell culture medium, overall throughput is greatly increased. Moreover, mammalian cells have little effect on assay performance compared with purified luciferase diluted into the same culture medium (Figure 2).

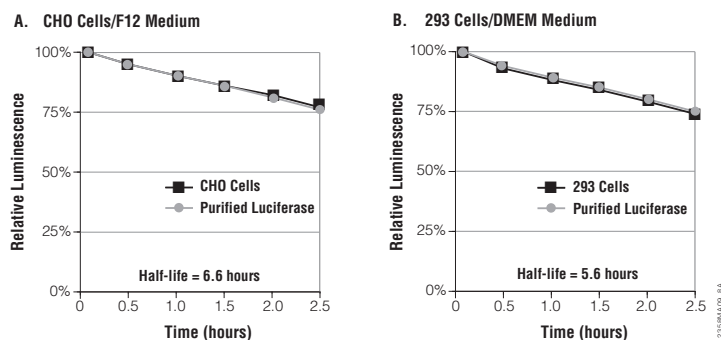


Figure 2. Reaction kinetics for purified firefly luciferase and luciferase expressed by stably transfected mammalian cells. Samples in 96-well plates consisted of either 100µl of purified enzyme (2.2×10^{-12} M with 1mg/ml BSA) or mammalian cells ($\sim 3 \times 10^5$ per well) that are stably transfected with the luciferase reporter gene. **Panel A.** CHO cells in F12. **Panel B.** HEK293 cells in DMEM. Luminescence measurements were integrated over 1 second per well. As these data show, very little difference in changes in relative luminescence over time is seen between transfected cells and purified enzyme in the same culture medium.

Broad applicability of the Steady-Glo® Reagent is an essential design criteria for generalized use in high-throughput applications. The reagent is compatible with the most commonly used culture media for mammalian cells (RPMI 1640, MEMα, DMEM and F12, with or without added serum) and can tolerate phenol red and organic solvents (Section 5.B). Furthermore, the reagent is tolerant of incomplete mixing, making it suitable for both 96- and 384-well plates (see next section, Improved Assay Precision). These performance criteria were maintained while maximizing luminescence intensity to achieve the highest possible assay sensitivity.

The physical characteristics of the Steady-Glo® Reagent were developed to achieve maximum efficiency and reproducibility. Because temperature is an important factor in assay performance (Section 5.B), the Steady-Glo® Luciferase Assay Buffer was formulated for stability at room temperature. Using the Steady-Glo® Luciferase Assay Buffer at room temperature allows preparation of Steady-Glo® Luciferase Assay Reagent that is ready for use immediately, without the need for temperature equilibration. As well, use of Steady-Glo® Luciferase Assay Reagent at room temperature ensures good assay performance and reproducibility. Other physical properties, such as density, viscosity and foaming activity, also were optimized for maximum assay reproducibility and compatibility with standard laboratory equipment.

Although the Steady-Glo® Luciferase Assay System yields a signal half-life of more than 5 hours, this is sometimes misinterpreted as providing signal stability for 5 hours. Half-life specifically refers to the rate of change in the signal (i.e., the time required for a 50% decrease in intensity). A half-life of greater than 5 hours equates to more than 87% of the signal remaining per hour. The total length of time that this signal is useful depends on the experimental design and conditions. Most applications require only 1–2 hours to measure luminescence from a stack of plates. Furthermore, as described previously, the precision of most experiments can be improved by including a control common to each plate being measured.

Improved Assay Precision

The Steady-Glo® Reagent provides greater assay precision through a novel formulation, making it more tolerant of variations in reactant concentrations (Figure 3). Because of this tolerance, deviations in luminescence between sample wells caused by incomplete mixing, pipetting errors or sample evaporation are minimized. This is particularly important for high-throughput processing of 96- and 384-well plates, where accurate pipetting and sample mixing may be difficult to achieve.

5.A. Overview of the Steady-Glo® Luciferase Assay System (continued)

Luminescence from luciferase assays designed for maximum sensitivity (i.e., maximum luminescence intensity) decays too quickly for batch processing of multiwell plates. Although the details are not understood, this decay is due to slow degradation of the enzyme during catalysis. To reduce the rate of degradation, thereby providing a more stable luminescent signal, the rate of catalysis also must be reduced. Hence, steady-state luciferase assays for high-throughput have lower luminescence intensities, resulting in lower assay sensitivities. This trade-off in sensitivity is generally accepted due to the premium placed on sample throughput.

Although lower catalytic rates can be achieved simply by adding an enzymatic inhibitor, the assay becomes susceptible to slight variations in the assay reagent. Specifically, enzyme activity (and light output) may be too low if the inhibitor concentration is too high. Conversely, activity may be too high if the inhibitor concentration is too low, leading to rapid decay of the enzyme and lower signal stability. This behavior is evident in conventional extended-lifetime luciferase assay reagents (Figure 3) and can reduce experimental precision under standard laboratory conditions.

The formulation of the Steady-Glo® Reagent largely overcomes the concentration effect of enzyme inhibition. Luminescence intensity of the assay is little changed over a wide range of reagent concentrations, making the reagent less sensitive to pipetting and mixing conditions. This characteristic allows good precision to be achieved more easily with automated liquid-handling equipment, potentially reducing the occurrence of false positive or false negative results 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.

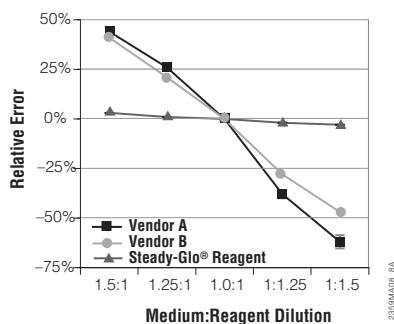


Figure 3. Effects of reagent concentration on assay precision. Purified firefly luciferase (2.2×10^{-10} M in RPMI 1640 with 1mg/ml BSA) was added to a 96-well plate at 100µl per well. Either Steady-Glo® Reagent or conventional extended-lifetime reagents were added to create the indicated dilutions. Luminescence measurements were integrated over 0.5 seconds per well. Relative error was calculated as the percent change of luminescence from that produced by the 1:1 dilution sample.

5.B. Conditions Affecting Assay Performance

The data presented in this section are intended to provide a general overview of assay characteristics under a wide range of experimental conditions. Note that the Steady-Glo® Luciferase Assay System is chemically different from other luciferase assays designed for high-throughput analysis. As a result, the data presented here may not be applicable to other luciferase assay systems.

Purified luciferase diluted in culture medium was used to generate much of the data presented in this section. This was done to illustrate performance characteristics of the reagent while avoiding experimental complexities common to cell culture. However, as demonstrated in Figure 2, purified luciferase diluted in culture medium shows little or no difference from enzyme expressed in transfected cells. BSA (1mg/ml) was added to the wells to simulate protein that would normally be contributed by the cells. However, addition of BSA is not required when using Steady-Glo® Reagent.

Culture Medium

In the Steady-Glo® Assay, culture medium and any other compounds contained therein comprise half of the chemical environment that defines the luminescent reaction. Although the Steady-Glo® Reagent is designed to work in conjunction with many common culture media, compositional differences between these different media can affect assay characteristics (e.g., light intensity and signal stability).

The Steady-Glo® Luciferase Assay System is designed to provide relatively high luminescence with signal half-lives greater than 5 hours with commonly used growth media. However, performance differences are evident between these media (Figure 4), as well as between different manufacturers of the same media. Sometimes, differences are also evident between lots of media from the same manufacturer. Although these differences are generally small and do not diminish the utility of the Steady-Glo® Assay, controls should be incorporated into every batch of plates to correct for this variability.

5.B. Conditions Affecting Assay Performance (continued)

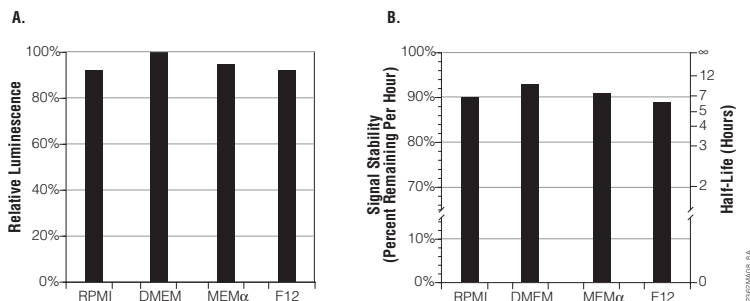


Figure 4. Relative intensity and signal stability of firefly luciferase in four common media. Purified firefly luciferase (2.2×10^{-10} M in medium with 1mg/ml BSA) was added to a 96-well plate at 100 μ l per well. Dilutions were made in RPMI 1640, DMEM, MEM α or F12 medium. Steady-Glo[®] Reagent was added, and luminescence measurements were integrated over 0.5 seconds per well. **Panel A.** Luminescence is shown relative to the light output generated in DMEM. **Panel B.** Signal stability in different media expressed as percent remaining per hour and as half-life. Number of samples = 4; relative standard error \leq 1.7%.

Serum

Steady-Glo[®] Luciferase Assay System is compatible with serum in medium. The Steady-Glo[®] Reagent is designed to be used with serum concentrations of 0-10%, and the luminescence signals generated are minimally affected by the presence of fetal bovine or calf sera (Figure 5).

Buffered Saline

Although luminescence measurements are generally performed in the culture medium in which the cells were grown, sometimes conditions require that the assay be performed using buffered saline (10mM buffer [pH 7.2-7.4], 150mM NaCl, 0.1mM MgSO₄). As illustrated in Table 1, different buffers produce luminescent reactions with varying light intensities and signal stabilities. Culture media generally provide a better combination of high luminescent intensity and long signal stability than the physiological buffers that have been tested with Steady-Glo[®] Reagent.

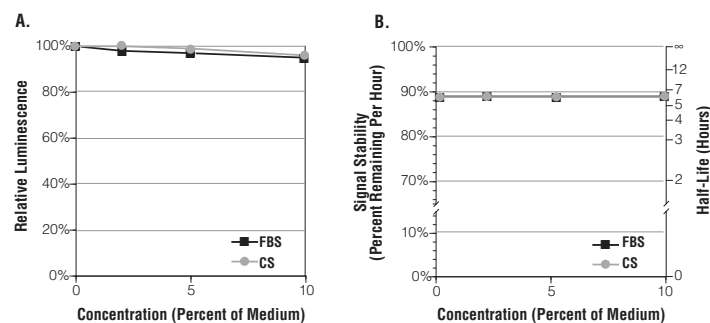


Figure 5. Effects of sera on intensity and signal stability. Purified firefly luciferase (2.2×10^{-10} M in medium with 1mg/ml BSA) was added to a 96-well plate at 100 μ l per well. DMEM containing various concentrations of fetal bovine serum (FBS) or calf serum (CS) was used for the assay. Steady-Glo[®] Reagent was added, and luminescence measurements were integrated over 0.5 seconds per well. **Panel A.** Luminescence is shown relative to that measured without serum. **Panel B.** Signal stability at various concentrations of sera expressed as percent remaining per hour and as half-life. Number of samples = 4; relative standard error \leq 0.9%.

Table 1. Luminescence Measured in Buffered Saline Solutions.

Buffer/Medium	% Relative Luminescence	% Relative Standard Error	% Remaining per Hour
RPMI 1640	100	0.3	93
Phosphate (Dulbecco's PBS)	56.7	0.8	91
HEPES	45.9	0.3	92
Glo Lysis Buffer	72.8	0.2	90

Purified firefly luciferase (2.2×10^{-10} M with 1mg/ml BSA) was added to a 96-well plate at 100 μ l per well. Dilutions were made in Dulbecco's PBS with 0.1mM MgSO₄ or 10mM HEPES (pH 7.4) with 150mM NaCl and 0.1mM MgSO₄ or RPMI 1640 or Glo Lysis Buffer (Cat.# E2661). Steady-Glo[®] Reagent was added, and luminescence measurements were integrated over 0.5 seconds per well. Luminescence is shown relative to that measured in RPMI 1640. Number of samples = 4.

5.B. Conditions Affecting Assay Performance (continued)

Phenol Red

Phenol red is a widely used pH indicator that is added to cell culture media. Many commercial medium formulations contain 5-15mg/L phenol red, evident by a red coloration in the medium. This compound will reduce assay sensitivity and slightly reduce signal stability (Figure 6). However, in most applications, the presence of phenol red will not significantly affect the utility of the Steady-Glo® Assay. To minimize its effect, use as little phenol red as possible in the culture medium.

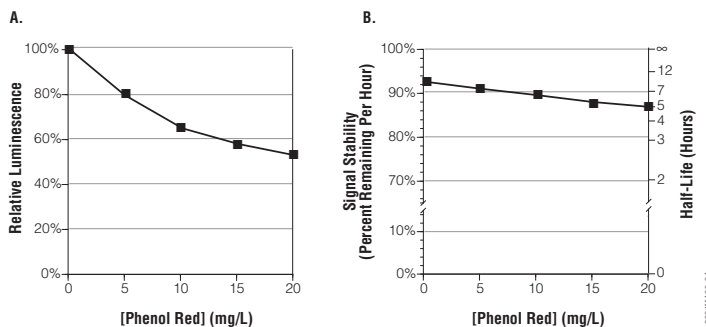


Figure 6. Effect of phenol red on light intensity and signal stability. Purified firefly luciferase (2.2×10^{-10} M in medium with 1mg/ml BSA) was added to a 96-well plate at 100 μ l per well. DMEM containing various concentrations of phenol red was used for the assay. Steady-Glo® Reagent was added, and luminescence measurements were integrated over 1 second per well. **Panel A.** Luminescence is shown relative to that measured without phenol red. **Panel B.** Signal stability at various concentrations of phenol red expressed as percent remaining per hour and as half-life. Number of samples = 3; relative standard error $\leq 0.7\%$.

Organic Solvents

Organic solvents are typically present in the luciferase assay, since they are used to stabilize screening compounds. At low concentrations, DMSO slightly increases assay sensitivity without significantly affecting signal stability. Isopropanol and ethanol have little effect on the assay (Figure 7). The compatibility of other solvents should be verified prior to use.

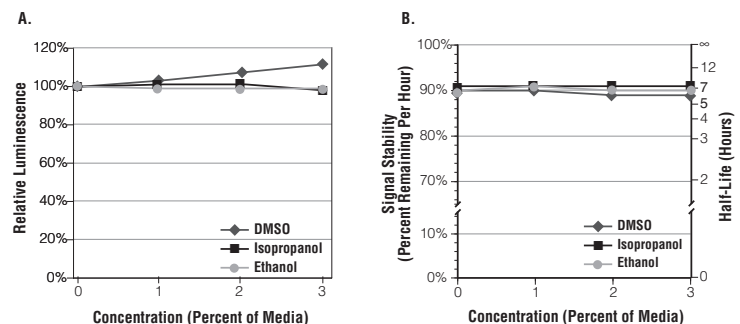


Figure 7. Effect of organic solvents on luminescence intensity and signal stability. Purified firefly luciferase (2.2×10^{-10} M in medium with 1mg/ml BSA) was added to a 96-well plate at 100 μ l per well. DMEM containing various concentrations of either DMSO, isopropanol or ethanol was used for the assay. Steady-Glo® Assay Reagent was added, and luminescence measurements were integrated over 0.5 seconds per well. **Panel A.** Luminescence is shown relative to that measured without organic solvents. **Panel B.** Signal stability at various concentrations of solvent expressed as percent remaining per hour and as half-life. Number of samples = 4; relative standard error $\leq 1.1\%$.

Medium Compositions

The Steady-Glo® Reagent is designed to work with the most commonly used culture media for mammalian cells. However, under some circumstances it may be desirable to use alternative medium formulations. Although it is difficult to predict precisely how these alternative media might affect performance of the Steady-Glo® Luciferase Assay System, the data below can be used as a guideline (Figure 8).

Figure 8 shows the effect of different concentrations of several components commonly used to define medium. Per graph, the concentration of each component is indicated for RPMI 1640, DMEM, MEM α and F12 media.

5.B. Conditions Affecting Assay Performance (continued)

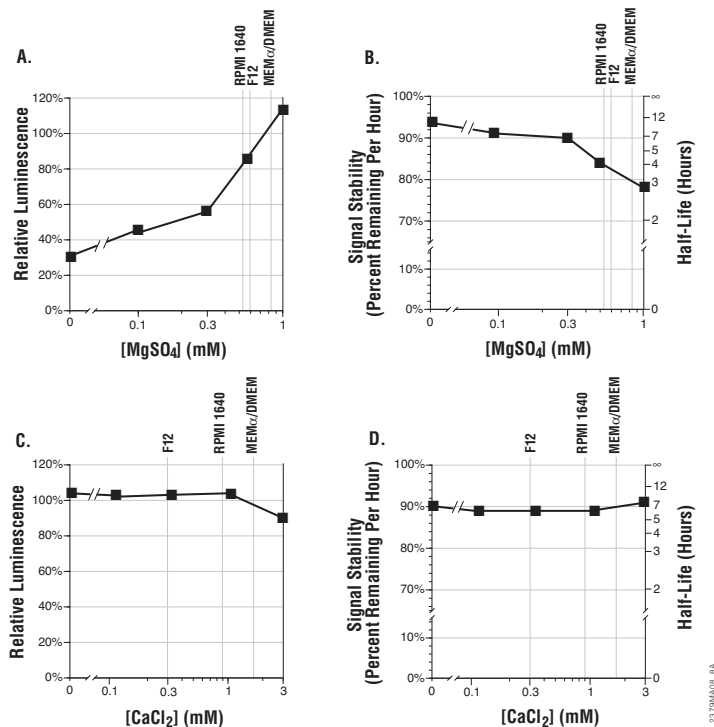


Figure 8. Effect of medium compositions on luminescence intensity and signal stability. Purified firefly luciferase (2.2×10^{-10} M in medium with 1mg/ml BSA) was added to a 96-well plate at 100 μ l per well. Each component (indicated on X axis) was tested in Dulbecco's PBS supplemented with 0.1mM MgSO₄. In those cases where the component is also a part of the buffered saline (i.e., NaCl and MgSO₄), the composition of the buffered saline was adjusted to yield the desired concentration range of the component being tested. For the phosphate titration, 10mM HEPES was used to buffer the solution at pH 7.4. Steady-Glo[®] Reagent was added, and luminescence measurements were integrated over 0.5 seconds per well. **Left Panels.** Luminescence is shown relative to that where the component concentration is equivalent to DMEM. **Right Panels.** Signal stability at various concentrations of each component expressed as percent remaining per hour and as half-life. At least three samples were used for each data point, and all relative standard errors were <1.7%.

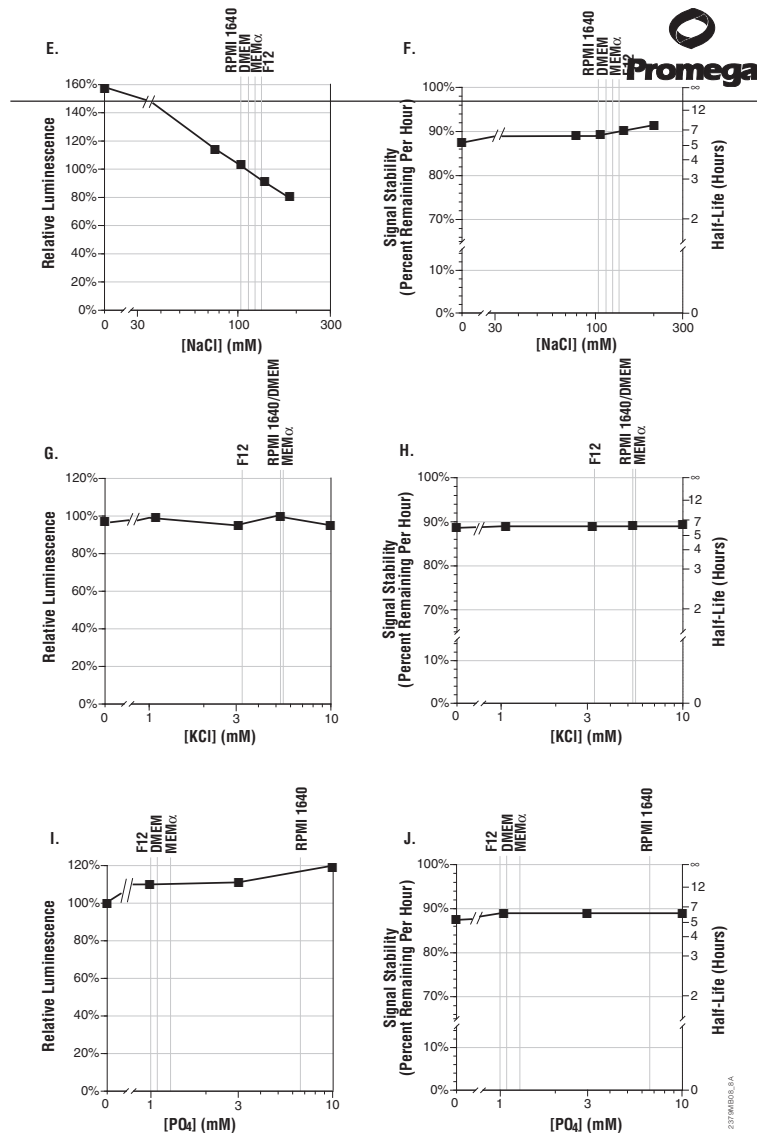


Figure 8. Effect of medium compositions on luminescence intensity and signal stability (continued).

5.B. Conditions Affecting Assay Performance (continued)

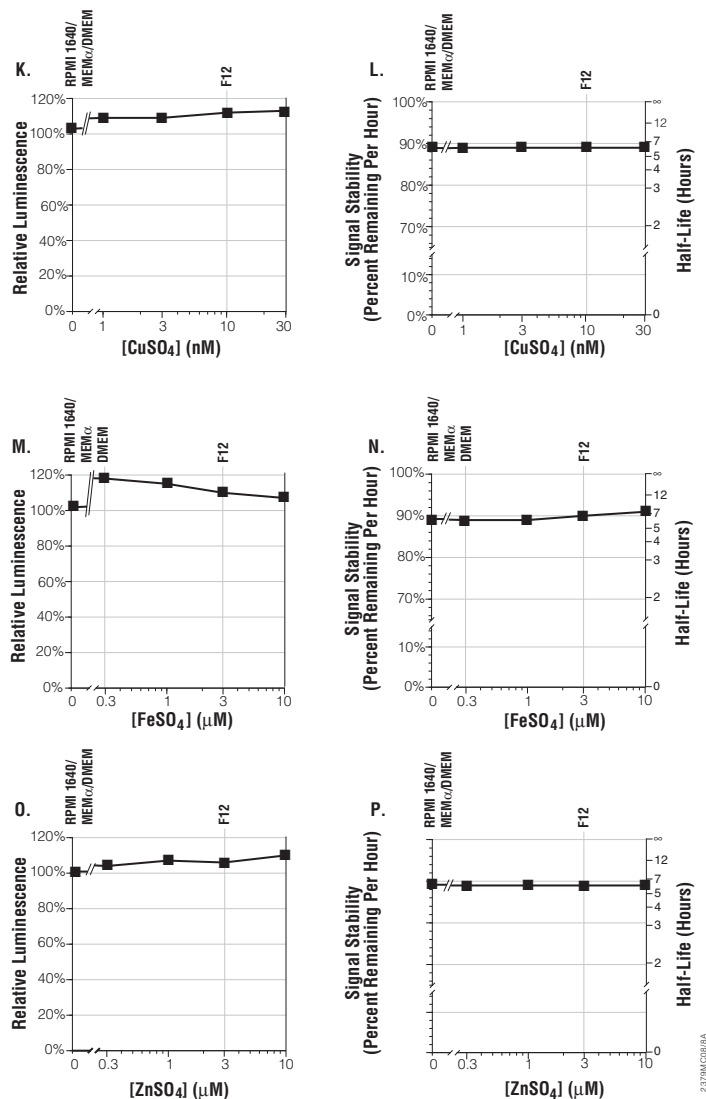


Figure 8. Effect of medium compositions on luminescence intensity and signal stability (continued).

Temperature

Since luciferase activity is temperature-dependent, temperature is an important factor in experimental precision (Figure 9). Good precision can be achieved most easily by performing all experiments at room temperature, as this is near the temperature optimum for luciferase activity. The Steady-Glo® Assay Reagent should be at room temperature before beginning measurements.

As mentioned previously, the Steady-Glo® Buffer can be stored at room temperature to avoid the need to temperature equilibrate the reagent before use. The heat capacity of the dried substrates is low; therefore, reconstitution of the Steady-Glo® Substrate with room-temperature Steady-Glo® Buffer yields Steady-Glo® Assay Reagent that is ready for use. If temperature equilibration is needed, use a water bath at room temperature for maximum heat transfer. Do not use a water bath above 25°C.

Lower temperatures result in increased signal stability, but luminescent intensity is lowered. If cold reagent is used, luminescence will slowly increase during the experiments as the reagent warms. High temperatures cause an increase in luminescence, but the signal becomes less stable. This can occur if the culture plates are too warm or if the luminometer produces excess heat within the reading chamber.

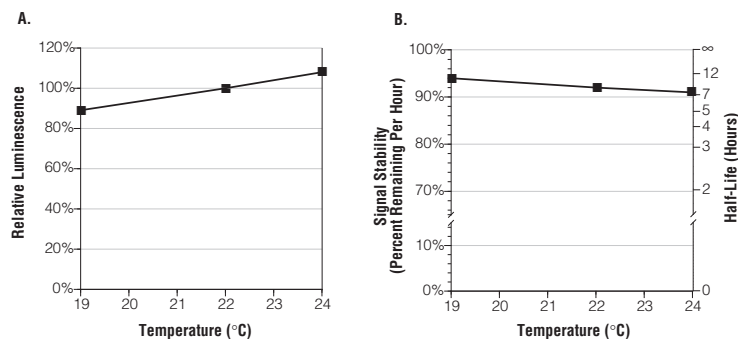


Figure 9. Effect of temperature on luciferase luminescence. Purified firefly luciferase (2.2×10^{-10} M in DMEM with 1mg/ml BSA) was added to luminometer tubes at 100μl per sample. Steady-Glo® Reagent was added, and reactions were stored at various temperatures. Luminescence was measured every 30 minutes for 2.5 hours (integrated over 5 seconds per sample). **Panel A.** Luminescence is shown relative to that measured at 22°C. **Panel B.** Signal stability at various temperatures expressed as percent remaining per hour and as half-life. Number of samples = 3; relative standard error $\leq 3.4\%$.

5.B. Conditions Affecting Assay Performance (continued)

Reagent Mixing

As described in Section 5.A, the Steady-Glo® Reagent minimizes the effect of concentration differences that can result from incomplete sample mixing. As with other performance criteria, this is affected by the culture medium composition (Figure 10). However, in all cases, the relative error caused by variable reagent concentration is much less than that of conventional extended-lifetime luciferase reagents (Figure 3).

Even though the effects of reagent concentration are minimized, good assay precision still depends on the efficiency of sample mixing. To produce the most uniform and reproducible data, the reagent and culture medium should be completely mixed prior to measurement. Where operational constraints make this impractical, optimization of sample-handling procedures can significantly affect data quality. This is particularly important for 384-well plates because of the small size of sample wells.

The most important handling consideration is the force and angle of reagent delivery into the wells of the plate. This can vary considerably depending on the brand and configuration of the pipette. Where reagent delivery is inefficient, adjusting the volume of reagent delivered or extending the incubation time before measurement can improve the results of the assay, as long as procedures are consistent and proper controls are included.

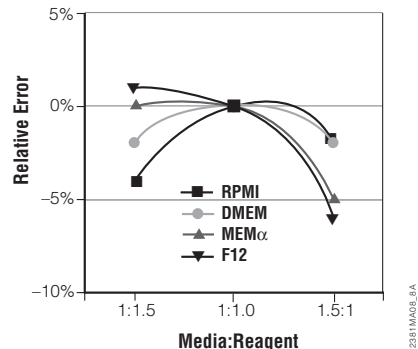


Figure 10. Effects of reagent concentration on assay precision. Purified firefly luciferase (2.2×10^{-10} M with 1mg/ml BSA) was added to a 96-well plate at 100 μ l per well. Steady-Glo® Reagent was added, and luminescence measurements in various dilutions of each assay reagent were integrated over 0.5 seconds per well for each medium. Relative error was calculated as the percent change of the luminescence from that produced by the 1:1 dilution sample. Number of samples = 4; relative standard error (per data point) $\leq 2.8\%$.

5.C. Procedure for Use with Glo Lysis Buffer

Glo Lysis Buffer, 1X, is a proprietary formulation that promotes rapid lysis (within 5 minutes) of cultured mammalian cells without the need for scraping or performing freeze-thaws of adherent cells. Glo Lysis Buffer is compatible with Steady-Glo® Assay Reagent to analyze firefly luciferase expression. Additionally, Glo Lysis Buffer is recommended as a diluent for firefly luciferase, because it provides enzyme stability of at least 2 days at room temperature (Figure 11). Similarly, in studies at Promega, firefly luciferase expressed in CHO, HEK293 and HeLa cells is stable after 48 hours at 22°C.

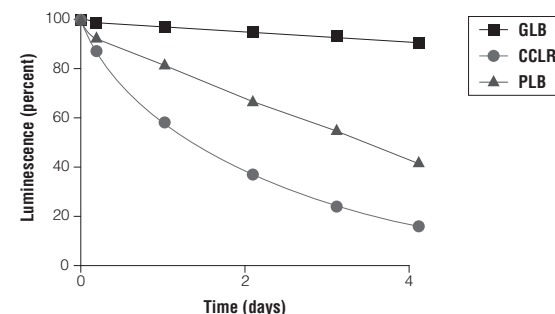


Figure 11. Stability of firefly luciferase in Glo Lysis Buffer. Purified firefly luciferase (2.2×10^{-10} M with 1mg/ml BSA) was added to Glo Lysis Buffer (GLB), Cell Culture Lysis Reagent (CCLR) or Passive Lysis Buffer (PLB). The three solutions were incubated at 22°C for up to 100 hours, with samples taken at 24-hour intervals and stored at -70°C. After 100 hours, all samples were thawed and initial luminescence measured. Luminescent reactions were initiated by adding 100 μ l of the respective luciferase solutions to Steady-Glo® Assay Reagent. Stability was determined as the amount of time necessary for initial luminescence to decrease to 90% of the luminescence at time zero.

5.C. Procedure for Use with Glo Lysis Buffer (continued)

Protocol

Glo Lysis Buffer can be used in a 1:1 ratio with reconstituted Steady-Glo® Assay Reagent following the protocol below.

1. Equilibrate the Glo Lysis Buffer to 22°C before use.
2. Equilibrate cells to room temperature. Aspirate medium from the cells. Gently rinse with 1X PBS (rinse is optional).
3. Add a sufficient volume of Glo Lysis Buffer to the sample well or plate to cover cells (Table 2). When using lysates for other applications, consider the volume needed for that application.
4. Rock the plate slowly several times to ensure complete coverage of cells with Glo Lysis Buffer.

Table 2. Amount of Glo Lysis Buffer for Use With Various Plate Sizes.

	Plate	Glo Lysis Buffer
	100mm	3ml
	60mm	1.1ml
	35mm	500µl
	6 wells	500µl
	12 wells	200µl
	24 wells	100µl
	96 wells	100µl

5. Incubate for 5 minutes at room temperature to allow cell lysis.
6. Transfer the lysate to luminometer tubes, plate wells or vials, and add a volume of Steady-Glo® Assay Reagent equal to the volume of Glo Lysis Buffer added. Wait 5 minutes, then measure luminescence with a luminometer, following the manufacturer's instructions.

Note: Lysate prepared with Glo Lysis Buffer can be stored at -20°C or -70°C and is stable over several freeze-thaw cycles.

Firefly Luciferase Enzyme Dilution Protocol with Glo Lysis Buffer

To prepare a positive control using QuantiLum® Recombinant Luciferase (Cat.# E1701), dilute the enzyme 10⁻⁶ in 1X Glo Lysis Buffer containing 1mg/ml BSA. Equilibrate the luciferase to room temperature for 20 minutes before performing an assay. Alternatively, the QuantiLum® Recombinant Luciferase can be diluted to 10⁻⁵ in 1X Glo Lysis Buffer containing 1mg/ml BSA, then diluted 1:10 in cell culture medium (final dilution of 10⁻⁶).

Note: Enzyme dilutions can be prepared and stored for up to 3 months at -80°C. Prepare enzyme by diluting in Glo Lysis Buffer to 10⁻³ to 10⁻⁶. Store in 500µl aliquots.

To perform a firefly luciferase activity assay, add 100µl of Steady-Glo® Assay Reagent to 100µl of QuantiLum® Recombinant Luciferase, diluted as indicated above. Wait 5 minutes, then read the sample in a luminometer.

5.D. References

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^(a)U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289, European Pat. No. 0 553 234 and Japanese Pat. No. 3171595.

^(b)The method of recombinant expression of *Coloptera* 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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