Dual-Glo™ Luciferase Assay System: Convenient Dual Reporter Measurements in 96- and 384-Well Plates

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Abstract

Promega has developed a new homogeneous reporter assay, the Dual-Glo™ Luciferase Assay System, for monitoring both firefly and Renilla *luciferases expressed in mammalian cells in culture medium in 96- and 384-well plates. The new assay system is similar to the Dual-Luciferase® Reporter Assay System, except that cell lysis is integrated into the luminescence chemistry and the luminescence kinetics have been extended over several hours. The homogeneous assay format allows dual-reporter assays to be more easily and rapidly performed in multiwell plates by reducing sample processing requirements and eliminating the need for reagent injectors in the luminometer.*

Like the DLR™ Assay, the new Dual-Glo™ Assay allows sequential measurement of both firefly and *Renilla* luciferases from one sample. However, the kinetics of the luminescence signals have been significantly extended.

Introduction

Although reporter genes are widely used for rapid evaluation of cellular physiology (1,2), a single reporter may not convey sufficient information for reliable interpretation of the experimental data. For this reason, dual reporters are commonly used, most notably the bioluminescent firefly and *Renilla* luciferase genes. Several years ago, Promega introduced the Dual-Luciferase® Reporter Assay System[a,b,c] (DLR™ Assay) to allow convenient sequential measurement of both reporters from a single sample (3). This assay was designed to provide optimal sensitivity and convenience for general research applications. However, when working with 96- or 384-well plates, the kinetics of the DLR™ Assay are not suited for quantitation in luminometers lacking integrated reagent injectors. Moreover, in high-throughput screening applications, which can involve analysis of well over 10,000 samples, it is necessary to keep sample processing requirements to a minimum.

Promega recently introduced the Dual-Glo™ Luciferase Assay System[a,b,c] (Cat.# E2920, E2940, E2980) for dual-reporter measurements in a homogeneous assay format. Like the DLR™ Assay, the new Dual-Glo™ Assay allows sequential measurement of both firefly and *Renilla* luciferases from one sample (Figure 1). However, the kinetics of the luminescent reactions have been

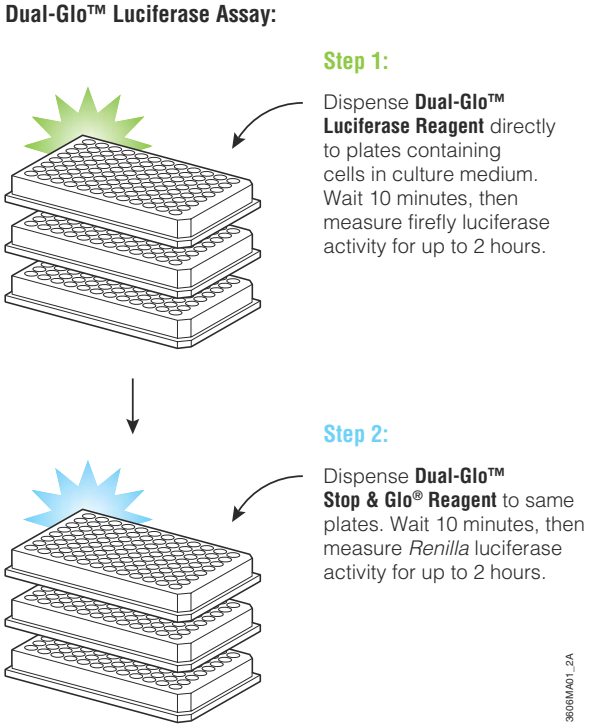

Figure 1. Schematic of the Dual-Glo™ Luciferase Assay method.

significantly extended to allow plenty of time for reagent addition to multiple samples before initiating measurements. Reagent can be added to all samples in a multiwell plate, or even to a stack of multiwell plates, before placing the plates in a luminometer. To further simplify sample processing, the lytic components of the assay have been combined with the luminescent chemistry to yield an integrated assay formulation. Consequently, the Dual-Glo™ Assay can be performed simply by adding the reagents directly to cells in culture medium and measuring the resulting luminescence.

Reporter genes offer an excellent means for extracting information from the complex genetic regulatory networks within cells. However, this complexity can also make it difficult to successfully isolate and characterize a specific physiological pathway without interferences from other elements within the system. The significance of these interferences on reporter responses can be realized only through a properly configured reference, typically from a secondary reporter. Dual reporters can thus facilitate the extraction of useful data by differentiating genetic responses of interest from non-relevant influences in the experimental system.

Interferences can arise from numerous and unidentified sources and thus are often recognized simply as "noise". The amount of experimental noise can be relatively large due to the inherent complexity of living cells. In other cases, the interference may result from known effects associated with the experimental method or biological system. Typical causes associated with the experimental method include so-called "edge effects" in multiwell plates or transfection efficiency in transiently transformed cells. Causes associated with the biological system occur when two pathways intersect, for example, when two receptor pathways use common intracellular components.

Interferences in the reporter response can also arise when the underlying genetic events are masked by other physiological factors such as cell viability. This is particularly problematic for distinguishing genetic downregulation from cytotoxicity.

For all these conditions, the DLR™ and Dual-Glo™ Assays provide rapid and convenient means for achieving greater control over the biological significance of reporter data. The Dual-Glo™ Assay adds new functionality by facilitating dual-reporter measurements for large numbers of samples in multiwell plates. In this regard, the Dual-Glo™ Assay is particularly well-suited for laboratory automation and high-throughput screening applications.

lab fact

Even greater Dual-Glo™ Assay sensitivity and reliability can be achieved when the assay is used in combination with the synthetic *Renilla* luciferase genes found in Promega's phRL and phRG Vectors.

Development of a Homogeneous Dual-Reporter Assay

Firefly and *Renilla* luciferases offer a nearly ideal dual-reporter system because both enzymes are readily and sensitively quantified. Both are immediately active upon mRNA translation, and as neither is naturally present in mammalian cells, their assays are not interfered with by endogenous enzymatic activities. As a dual-reporter system, these enzymes also offer the advantage of having completely separate evolutionary histories and thus independent biochemistries, allowing their luminescent reactions to be distinguished.

The DLR™ Assay enables quantitation of both reporters in a cellular lysate through sequential addition of two complementary reagents. The first reagent added to the lysate initiates the firefly reaction. After quantifying the firefly luminescence, the second reagent is added to initiate the *Renilla* reaction while simultaneously quenching the firefly luminescence. The luminescence of this second reaction is then also quantitated. Both reagents in the DLR™ Assay have been optimized for maximum sensitivity.

However, because luciferases are subject to catalytically induced auto-inactivation, the duration of the luminescent signals is relatively short. For the firefly reaction, the luminescence decreases by 50% in approximately 12–15 minutes. The *Renilla* reaction is even more brief, reducing by 50% in less than 3 minutes. While these reaction kinetics are suitable for routine measurement of a small number of samples, they are problematic for analysis in 96- and 384-well plates. Because most luminometers require several minutes to read an entire plate, the adaptation of integrated reagent injectors is normally necessary. Simply adding assay reagents to the plate before reading would produce an unacceptable loss of luminescence before the last well was measured. Although onboard reagent injectors are suitable for analysis in 96-well plates, they can significantly reduce sample throughput. The efficacy of reagent injectors for 384-well plates is questionable.

Because the DLR™ Assay was designed for analysis of cell lysates, some sample processing is required before the assay can begin. The lysate must be prepared by removing the culture medium and adding a lysis reagent to each sample. Although this may be feasible for a small number of multiwell plates, it can be overly cumbersome for larger sample sets. Due to these requirements, the DLR™ Assay is especially problematic for implementation by laboratory automation. Although robotic systems can be designed to separate cells from medium and compensate for the relatively short reaction kinetics, the greater hardware complexity increases costs and processing failures.

As laboratory automation is increasingly important for reporter analysis, the new Dual-Glo™ Assay was

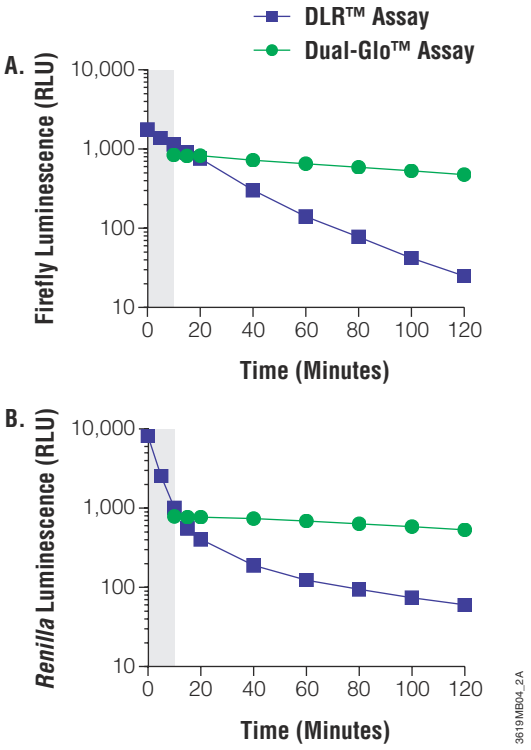

Figure 2. Luminescence kinetics of the Dual-Glo™ and DLR™ Assays. Firefly and *Renilla* luciferases were diluted to a final concentration of 1.67×10^{-9}M (plus 1mg/ml gelatin) and assayed based on the recommended procedure in the Technical Manuals (#TM058 and #TM040, respectively). For the Dual-Glo™ Assay, the luciferases were diluted into culture medium (RPMI 1640) and luminescence measured 10 minutes after addition of the assay reagents. For the DLR™ Assay, the luciferases were diluted into Passive Lysis Buffer (Cat.# E1941). Luminescence measurements were initiated immediately upon reagent addition. Measurements for both assays were made repeatedly over 2 hours. The results reveal stable luminescence for both the firefly **(Panel A)** and *Renilla* **(Panel B)** luciferases when using the Dual-Glo™ Assay, with comparable luminescence intensities for both luciferases.

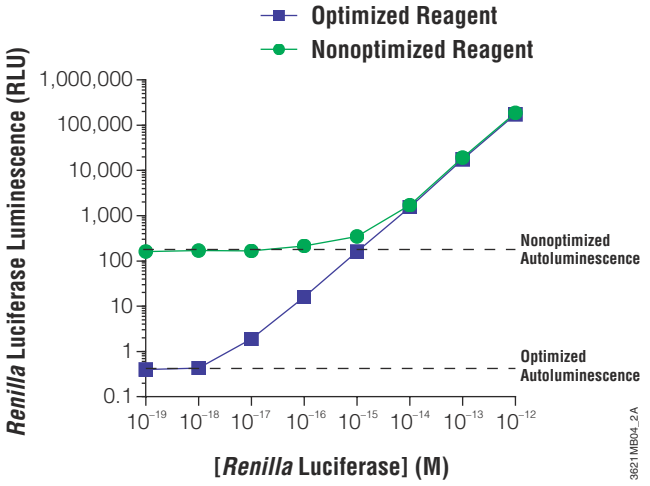

Figure 3. Dual-Glo™ Assay minimizes background luminescence. A titration of *Renilla* luminescence, over a range of 1×10^{-12} to 1×10^{-19} moles/reaction in RPMI 1640 medium, was performed using the fully optimized Dual-Glo™ Assay or an assay prototype not formulated to control autoluminescence. Assays were performed on 100µl samples using a Turner Designs Model 20e Luminometer; measurements were made 10 minutes after reagent addition at 22°C. The results show that the optimized assay greatly increases sensitivity by lowering the limit of detection (dashed lines indicate three standard deviations above the respective backgrounds).

designed to overcome the limitations of the DLR™ Assay. It is especially suited for high-throughput quantitation of mammalian cells grown in 96- or 384-well plates. The assay system allows reagent to be added directly to cells in culture medium without requiring separation or washing steps. The first assay reagent initiates the firefly luminescence for quantitation and also lyses the cells. The second reagent initiates the *Renilla* luminescence and quenches the firefly luminescence by greater than 10,000-fold. Thus, luminescence "bleed-through" between the sequential reactions is virtually eliminated, even if the firefly reporter is 100-fold brighter than the *Renilla* reporter. The luminescence kinetics of both reporters is greatly extended relative to the DLR™ Assay, decreasing less than 50% in 2 hours (Figure 2).

The design of the Dual-Glo™ Assay greatly simplifies the experimental requirements for dual-reporter measurements in multiwell plates. Due to the extended luminescence kinetics, assay reagent may be added to all plate wells with little concern for timing. Even if serial addition of reagent requires up to 10 minutes to complete, this would produce a difference of only a few percent in the relative luminescence values. In applications requiring quantitation of many plates, incorporating one or more control wells in each plate can compensate for the gradual change in reporter luminescence. This minimizes the temporal separation between samples and controls and allows accurate comparisons between samples located on different plates. Because the *Renilla* luciferase is stable after addition of the first reagent, the timing for addition of the second reagent is not critical and can follow the first reagent by several hours.

The extended luminescence kinetics of the Dual-Glo™ Assay are achieved by reducing catalytic turnover of the enzymes, which correspondingly reduces assay sensitivity. Higher catalytic turnover in the DLR™ Assay yields greater sensitivity, but also drives the unavoidable auto-inactivation at a higher rate. However, in most applications the reduced sensitivity of the Dual-Glo™ Assay is an acceptable compromise. The high sensitivity of the DLR™ Assay is generally excessive in common experimental systems, even for the small samples contained in 384-well plates.

Sensitivity of *Renilla* luciferase measurements could be adversely affected by non-enzymatic background luminescence caused by spontaneous oxidation of the enzyme substrate. This background luminescence, termed "autoluminescence", is greatly enhanced by hydrophobic microenvironments as can be provided by detergents. In the DLR™ Assay, a specially designed lysis reagent limits the effect of detergent-enhanced

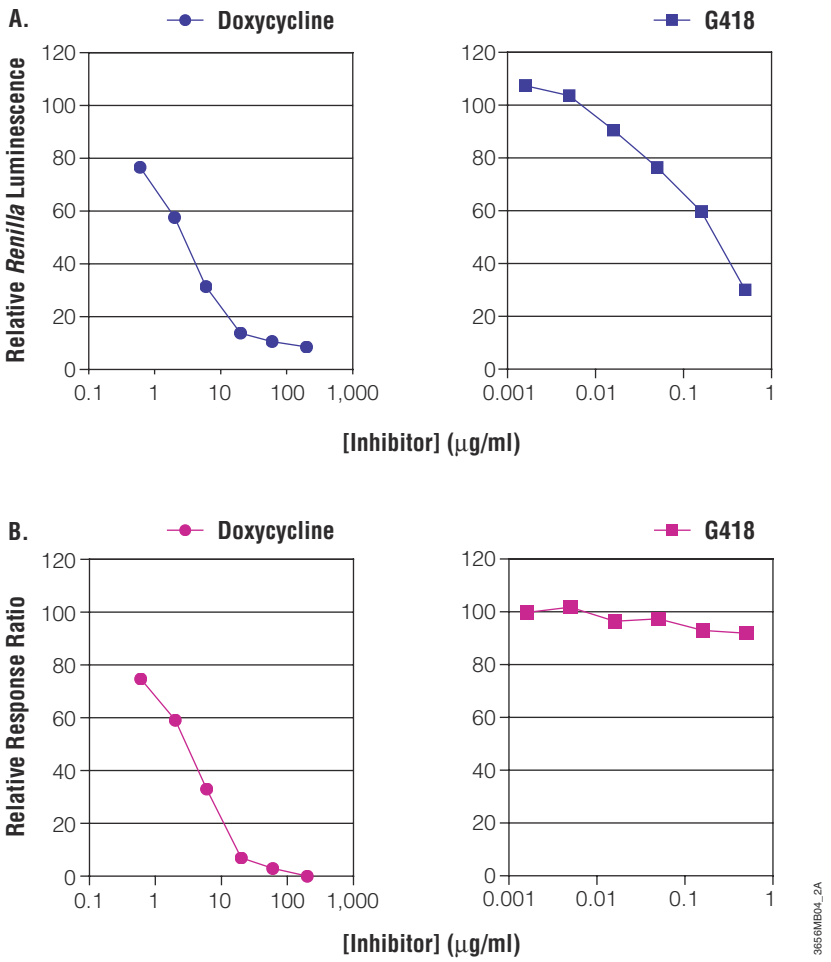

Figure 4. Differentiating genetic downregulation from cytotoxicity. CHO cells were transiently transfected with *Renilla* luciferase under the control of the Tet-Off promoter and firefly luciferase under the control of the CMV promoter. Cells were titrated with a specific inhibitor of *Renilla* luciferase expression (doxycycline) or a cytotoxic agent (G418 antibiotic). **Panel A** illustrates that the output from a single reporter is similar under both inhibitors, as the test compounds both yield diminished reporter luminescence. The y axis shows relative *Renilla* luminescence as a percent of sample without inhibitor. In contrast, **Panel B** shows that a dual-reporter system can clearly distinguish between specific downregulation of the gene and cytotoxicity. The output is recorded as a relative response ratio (RRR), where the sample, negative and positive controls are measured as the ratio of *Renilla* luminescence to firefly luminescence. Measurements of the relative response ratios were also much more precise than the measurements of a single reporter (average relative standard deviation of 6.5% in Panel B compared with 13% in Panel A).

$$RRR = \frac{[\text{sample} - \text{negative control}]}{[\text{positive control} - \text{negative control}]}$$

autoluminescence. However, to integrate cell lysis into the homogeneous format of the Dual-Glo™ Assay, a much higher concentration of detergent was required, leading to greater inherent autoluminescence. To overcome this problem, a novel formulation strategy was used that reduces autoluminescence by greater than 100-fold (Figure 3; 4).

Increasing Data Reliability

When a specific interfering event dominates, its influence can lead to misinterpretation of experimental results. This can occur, for example, when cytological perturbations are misconstrued as changes in genetic regulation. Particularly when using specific downregulation as a means of identifying novel receptor antagonists, a reduction of reporter expression can be confused with cytotoxicity. Again, this situation can generally be remedied by using a second genetic reporter.

To model this circumstance, dual-reporter measurements were made in cells expressing *Renilla* luciferase under control of the Tet-Off promoter and firefly luciferase under control of the CMV promoter (Figure 4). Reporter activity was measured after adding titrated amounts of either doxycycline or G418 antibiotic to the cells. Doxycycline is expected to specifically downregulate the *Renilla* expression coupled to the Tet-Off promoter, while G418 is expected to kill the cells. As both compounds reduce *Renilla* luminescence with increasing dose, it is not possible to distinguish specific genetic regulation from cell death using a single reporter. In contrast, by using the firefly luciferase as an internal reference, the distinction between genetic response and cell death is

clear. Doxycycline reduces only *Renilla* luminescence, whereas cell death caused by G418 reduces the luminescence of both reporters. This distinction can be readily displayed as the ratio of *Renilla* to firefly luminescence.

Significant genetic reporter responses can also be masked by the statistical uncertainty of experimental noise. This noise can result from countless and often uncontrollable events, including inconsistent transfection efficiencies, inconsistent cell dispensing, temperature or humidity gradients (i.e., "edge effects"), incubator vibrations, etc. Various metrics of precision, such as standard deviation, coefficient of variance, and Z'-factor (5), are commonly used to estimate the effect of this noise on assay reliability.

To reduce the influence of noise, a second reporter is commonly used as an internal control (6). Since most nonspecific events will similarly influence both the primary and control reporters, the ratio of their responses is more robust to such interference. Hence, the ratio of dual-reporter assays is typically more precise than either assay alone. For example, in the model described above, the average relative standard deviation of the *Renilla* luciferase measurements was 13%, while the average relative standard deviation of the response ratio for both reporters combined was only 6.5% (Figure 4).

Conclusions

Experimental strategies involving dual reporters have become increasingly prevalent to provide reliable and meaningful data. Reporter analyses also are increasingly being performed in multiwell plates to enable more convenient processing of more samples. The culmination of these trends is high-throughput screening, where huge numbers of samples are quantitatively analyzed. The Dual-Glo™ Assay System was developed to support these requirements by providing a simple homogeneous means of quantifying both the firefly and *Renilla* luciferases from mammalian cells in culture medium.

References

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Protocols

- *Dual-Glo™ Luciferase Assay System Technical Bulletin* #TM058, Promega Corporation.
  www.promega.com/tbs/tm058/tm058.html

Ordering Information

Product	Size	Cat.#
Dual-Glo™ Luciferase Assay System	10ml	E2920
	100ml	E2940
	10 × 100ml	E2980

[a] Certain applications of this product may require licenses from others.

[b] U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289, 5,814,471, Australian Pat. No. 649289 and European Pat. No. 0 553 234 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. Other patents are pending.

[c] U.S. Pat. No. 5,744,320 and Australian Pat. No. 721172 have been issued to Promega Corporation for quenching reagents and assays for enzyme-mediated luminescence. Other patents are pending.


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