

Dual-Luciferase™ Reporter Assay: An Advanced Co-Reporter Technology Integrating Firefly and *Renilla* Luciferase Assays

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In the quantitation of gene expression using firefly luciferase, a second reporter gene is commonly used to minimize experimental variability. However, traditional co-reporters (e.g., CAT, beta-Gal or GUS) are inconvenient due to differences in their respective assay chemistries, handling requirements and measurement characteristics. Promega introduces a superior dual-reporter technology integrating the assay of firefly luciferase with the Renilla luciferase assay. The Dual-Luciferase™ Reporter Assay System, in combination with the pRL Vectors which express Renilla luciferase as the second reporter, offers the exceptional speed, sensitivity and convenience of two luciferase reporter assays in a single-tube format. The system also includes Passive Lysis Buffer, formulated to provide quantitative solubilization of both luciferases from mammalian cells cultured in multi-well plates without the need for individual processing of each sample. The Dual-Luciferase™ Reporter Assay System will provide immediate benefit to researchers currently using any experimental firefly luciferase reporter vector.*

*Patent pending.

Introduction

Dual-reporters are used to make relational or ratiometric measurements within an experimental system. Typically one reporter is used as an internal control to which measurement of the other reporter is normalized. In measurements of gene expression, dual reporters are generally used in transient transfections of cultured cells, where one vector containing the experimental reporter gene is co-transfected with a second vector containing a distinct reporter gene serving as the control. Usually, the experimental reporter is coupled to a regulated promoter to study the structural or physiological basis of regulated gene expression. Relative changes in the expression of reporter activity correlate to changes in the transcriptional activity of the coupled regulated promoter. To provide an internal control for transcriptional activity, the second reporter gene is coupled to a constitutive promoter that is unperturbed by the various experimental conditions. By this method, it is possible to minimize inherent variabilities that can undermine experimental accuracy, such as differences in the number and health of the cultured cells, and the efficiencies of cell transfection and lysis.

Dual-reporter applications utilizing firefly luciferase in combination with either chloramphenicol acetyltransferase (CAT), beta-galactosidase (beta-Gal) or beta-glucuronidase (GUS) have become popular in recent years. However, these co-reporter combinations diminish the performance advantages of luciferase. For example, while the luciferase assay can be performed and quantitated in seconds, the CAT, beta-Gal and GUS assays are endpoint assays requiring lengthy incubation periods prior to quantitation. Furthermore, these other reporters are limited in their sensitivity and in the range of their linear response; care must be taken not to exceed these ranges. Endogenous cellular activities can also interfere with the use of these reporters. Many cell types exhibit endogenous beta-Gal or GUS expression which hinder accurate quantitation of reporter gene expression, and endogenous deacetylase activity which interferes with assays of CAT activity. Although prior treatment of cell lysates at high temperature (1,2) can reduce the interfering endogenous activities for the beta-Gal and CAT assays, such treatment rapidly inactivates luciferase. Therefore, in dual-reporter measurements, this necessitates division and differential processing of co-transfected cell lysates prior to performing the reporter assays.

The ideal dual-reporter method would allow the user to assay both reporters in a single sample with the speed, sensitivity, and linearity achievable with firefly luciferase. This is impossible with traditional reporters like CAT, beta-Gal and GUS due to limitations inherent in their assay chemistries and handling requirements. In contrast, Promega's Dual-Luciferase™ Reporter (DLR) Assay System meets these demanding criteria by combining the assays of two luciferase reporter systems, those of the firefly (*Photinus pyralis*) and the sea pansy (*Renilla reniformis*), in an integrated, single-tube assay format.

Dual-Luciferase™ Reporter Assay chemistry

Although both the firefly and *Renilla* luciferases provide the superior assay characteristics of bioluminescent reporters, they possess distinct evolutionary origins and, therefore, have dissimilar enzyme structures and substrate requirements. These differences have allowed us to develop the DLR Assay chemistry that selectively discriminates between these two luminescent reporter activities.

Firefly luciferase is a 61kDa monomeric protein that does not require post-translational processing for enzymatic activity (3,4). Thus, it functions as a genetic reporter immediately upon translation. Photon emission occurs via oxidation of beetle luciferin in a reaction that requires ATP, Mg²⁺ and O₂ (Figure 1). Under conventional reaction conditions, the oxidation of luciferin occurs through a luciferyl-

AMP intermediate that turns over very slowly. As a result, this assay chemistry generates a "flash" of light that rapidly decays after the substrate and enzyme are mixed. Promega's patented assay reagents for quantifying firefly luciferase activity incorporate coenzyme A (CoA) to provide enhanced reaction kinetics by promoting rapid enzymatic turnover (5), resulting in an extended "glow" luminescence signal (Figure 2).

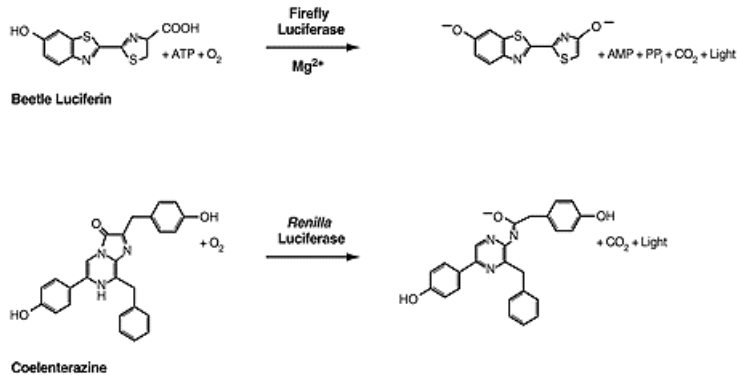


Figure 1. Bioluminescent reactions catalyzed by the firefly and *Renilla* luciferases.

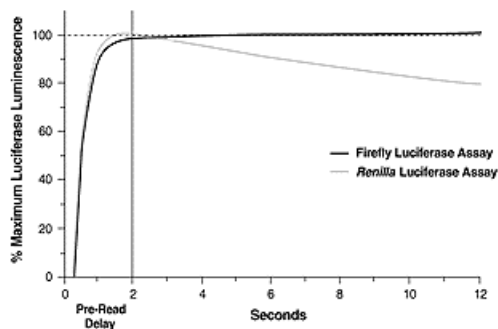


Figure 2. Luminescence generated by the firefly and *Renilla* luciferases using the Dual-Luciferase™ Reporter

Assay. CHO cells (1×10^6 cells/60mm culture dish) were co-transfected with pGL3-Control and pRL-SV40 Vector DNAs. Cells were washed with PBS and lysates prepared by applying 400 μ l of PLB and scraping. A 20 μ l aliquot of cell lysate was mixed with 100 μ l of Luciferase Assay Reagent II (LAR II) and firefly luciferase activity immediately measured with a luminometer (Green trace). 100 μ l of Stop & Glo™ Reagent was then added to the luminometer tube to quench the firefly luciferase reaction and simultaneously activate the *Renilla* luciferase reaction. *Renilla* luciferase activity was measured immediately (Blue trace). A Turner Designs Model 20e Luminometer interfaced with a computer was used to trace the luminescence emission over a 12 second period in both determinations.

Renilla luciferase, a 36kDa monomeric protein, is composed of 3% carbohydrate when purified from its natural source, *Renilla reniformis* (6). However, like firefly luciferase, post-translational modification is not required for activity and the enzyme may function as a genetic reporter immediately following translation. The luminescent reaction catalyzed by *Renilla* luciferase utilizes O₂ and coelenterate luciferin (coelenterazine) (Figure 1). When performed using the DLR Assay chemistry, the kinetics of the *Renilla* luciferase reaction provide a glow-type luminescent signal that decays slowly over the course of the measurement (Figure 2).

In the DLR Assay System, the activities of the firefly and *Renilla* luciferases are measured sequentially from a single lysate. Upon completing the measurement of firefly luciferase activity (the "experimental" reporter), the firefly luminescence is rapidly quenched, with simultaneous activation of the *Renilla* luciferase luminescent reaction (the "control" reporter activity). Thus, the DLR Assay System integrates the two assay chemistries to provide rapid quantitation of both reporters co-expressed in the lysates of transfected cells, or in cell-free transcription/translation reactions.

The linear range of the firefly luciferase assay extends over 8 orders of magnitude of enzyme concentration, providing detection of 1 femtogram (approximately 10^{-20} mole) of experimental reporter enzyme (Figure 3A). In the Dual-Luciferase™ Reporter Assay System, *Renilla* luciferase has a linear range over 7 orders of magnitude of enzyme concentration, with a lower detection limit of ≤ 10 femtograms (approximately 3×10^{-19} mole) of control reporter enzyme (Figure 3B). Furthermore, the apparent specific activities of the two enzymes are similar.

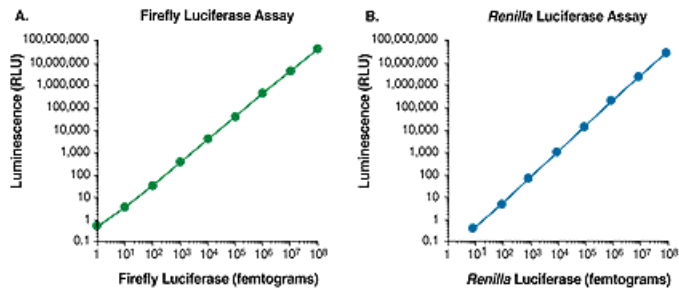


Figure 3. Linear range of firefly luciferase and *Renilla* luciferase luminescence reactions. Purified firefly and *Renilla* luciferases were serially diluted in Passive Lysis Buffer containing 1mg/ml BSA. The activities of both luciferases were determined at various dilutions using a Turner Designs Model 20e Luminometer to integrate light emission over a 10 second reaction period, after an initial 2 second pre-read delay.

Format of the Dual-Luciferase™ Reporter Assay

Quantitating the luminescent signal from the two luciferase reporter enzymes may be performed immediately following lysate preparation without the need for dividing samples or performing additional treatments. Because both *Renilla* and firefly luciferase exhibit glow-type reaction kinetics, performing the Dual-Luciferase™ Reporter Assay does not require a luminometer equipped with reagent injectors.

A typical DLR Assay, which requires about 30 seconds to complete, is depicted in [Figure 4](#). The firefly luciferase reporter assay is initiated by mixing an aliquot of lysate with Luciferase Assay Reagent II (LAR II). Upon completion of the firefly luciferase assay, the firefly luminescence is quenched and *Renilla* luminescence is simultaneously activated by adding Stop & Glo™ Reagent to the sample tube. The Stop & Glo™ Reagent quenches the luminescence signal from the firefly reaction by $>10^5$ -fold within 1 second ([Figure 5](#)). The simultaneous activation of *Renilla* luciferase is also achieved within this 1 second period.

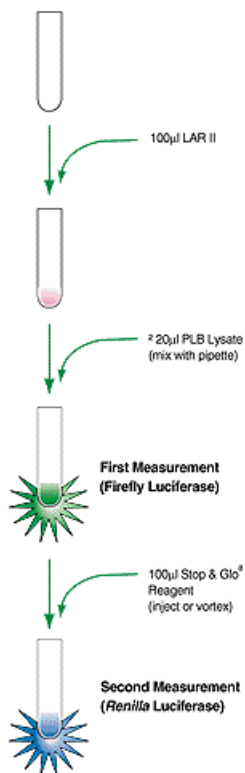


Figure 4. Format of the Dual-Luciferase™ Reporter Assay using a manual luminometer or a luminometer equipped with one reagent injector. If the luminometer is equipped with two injectors, pre-dispense the lysate samples into luminometer tubes, followed by sequential auto-injection of Luciferase Assay Reagent II (LAR II) and Stop & Glo™

Reagents.

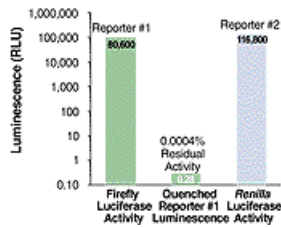


Figure 5. The Dual-Luciferase Reporter Assay System quenches firefly luciferase luminescence and activates *Renilla* luciferase luminescence. Lysates of CHO cells co-transfected with pGL3-Control and pRL-SV40 Vector DNAs were prepared as described in [Figure 2](#). Firefly luciferase (Reporter #1) and *Renilla* luciferase (Reporter #2) activities were measured over a 10 second reaction period, after an initial 2 second pre-read delay. To demonstrate the efficiency of quenching of Reporter #1 by Stop & GloTM Reagent, firefly luciferase luminescence was quenched without activation of *Renilla* luciferase luminescence by adding an equal volume of Stop & GloTM Buffer (which lacks coelenterazine and is therefore incapable of activating the *Renilla* luciferase reaction). In this experiment, the residual luminescence of the quenched firefly luciferase reaction was less than 0.0004% of the value of the unquenched reaction.

Passive Lysis Buffer

Passive Lysis Buffer (PLB) was specifically formulated to promote efficient lysis of cultured mammalian cells without the need for scraping adherent cells or performing freeze-thaw cycles. Although PLB is designed for passive lysis applications, its robust lytic performance is of equal benefit when lysates are prepared using conventional sample processing. Regardless of the lysis method, the release of firefly and *Renilla* luciferase reporter enzymes into the PLB lysate is both quantitative and reliable for cultured mammalian cells ([Figure 6](#)).

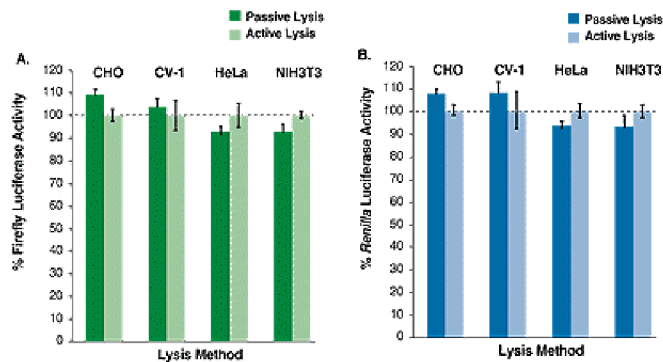


Figure 6. Efficiencies of mammalian cell lysis using PLB and either passive or active lysis methods. The indicated types of cultured mammalian cells were co-transfected with pGL3-Control and pRL-SV40 Vector DNAs. Lysates were prepared by exposing adherent cells to PLB and either gently rocking the cultures for 15 minutes (passive cell lysis) or scraping adherent cells from the culture dish and performing one freeze/thaw cycle at -80°C (active cell lysis). Activities for firefly and *Renilla* luciferases were determined using the DLR Assay, as outlined in [Figure 4](#). For comparative purposes, reporter activities were normalized to the active lysis method for each cell type. **Panel A:** Firefly luciferase reporter activity. **Panel B:** *Renilla* luciferase reporter activity.

A particular advantage of Passive Lysis Buffer is its suppression of the low level non-enzymatic luminescence (auto-luminescence) which is an inherent property of coelenterazine in aqueous solutions. Coelenterazine auto-luminescence is intensified by some reagents commonly used to prepare cell lysates, including Triton® X-100, and Promega's Cell Culture Lysis Reagent (CCLR) and Reporter Lysis Buffer (RLB). In addition, these lysis reagents can significantly inhibit the luminescence reaction of *Renilla* luciferase. PLB is specifically formulated to maximize luciferase activity and minimize auto-luminescence to provide optimal assay sensitivity, allowing quantitation of even low levels of *Renilla* luciferase activity ([Figure 7](#)).

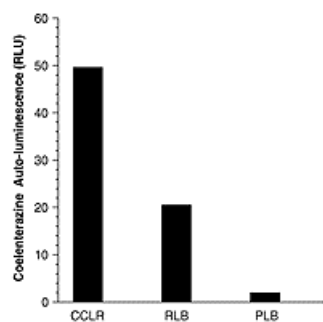


Figure 7. Influence of lysis buffer composition on coelenterazine auto-luminescence. Lysates of non-transfected control (NTC) CHO cells were prepared in either CCLR (Cat.# E1531), RLB (Cat.# E3971) or PLB, and aliquots assayed using the DLR Assay System as described in [Figure 2](#). Coelenterazine auto-luminescence was measured over a 10 second period after addition of Stop & GloTM Reagent (which contains coelenterazine) to the reaction mix. No measurable luminescence was detected in the absence of Stop & GloTM Reagent (data not shown).

In addition to these advantages, PLB supports the long-term stability of both the firefly and *Renilla* luciferase reporter enzymes in mammalian cell lysates ([Figure 8](#)). Furthermore, PLB suppresses sample foaming, making it ideally suited for high-throughput applications in which arrays of cells cultured in multi-well plates are processed into lysates and assayed using automated systems.

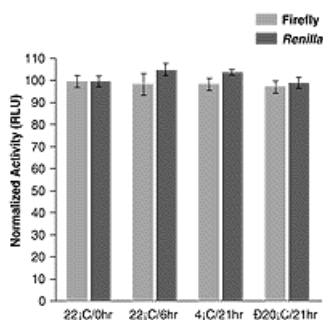


Figure 8. Stability of firefly and *Renilla* luciferases in cell lysates prepared with Passive Lysis Buffer. CHO cells cultured in standard 6 well culture plates (2.5 x 10⁵ cells/well) were co-transfected with pGL3-Control and pRL-SV40 Vector DNAs. After a 30 hour incubation, the culture medium was aspirated and adherent cells in each well were washed once with PBS. Lysates were prepared by applying 500µl of Passive Lysis Buffer to each culture well and incubating at room temperature with gentle rocking for 15 minutes. Aliquots of each lysate were immediately assayed for firefly and *Renilla* luciferase activities, then divided and stored at either 22°C (room temperature), 4°C, or -20°C. Stored samples were reassayed at the time intervals indicated. Assays were performed as described in [Figure 5](#); each bar represents the mean of triplicate cell lysates with duplicate assay measurements.

The pRL family of *Renilla* luciferase control vectors

The pRL Vectors are designed to provide constitutive expression of *Renilla* luciferase in mammalian cells. These vectors contain the *Renilla* luciferase cDNA* (*Rluc*) cloned from *Renilla reniformis* (7). Three base substitutions have been introduced into the *Rluc* gene to eliminate internal *Bgl* II, *Bam*H I and *Nar* I restriction sites. The amino acid sequence of the expressed *Renilla* luciferase is unchanged by these mutations. The pRL Vectors are available in several promoter configurations and may be used in combination with any experimental firefly luciferase vector to provide an internal control of reporter activity.

*The cDNA encoding luciferase from *Renilla reniformis* is the subject of U.S. Patent No. 5,292,658 assigned to the University of Georgia and sublicensed from SeaLite Sciences, Inc., Bogart, GA.

pRL-TK

The pRL-TK Vector ([Figure 9](#)) contains the herpes simplex virus thymidine kinase (HSV-TK) promoter region upstream of *Rluc*. The HSV-TK promoter provides low-level, constitutive expression in cells of both embryonal and mature mammalian tissues (8,9).

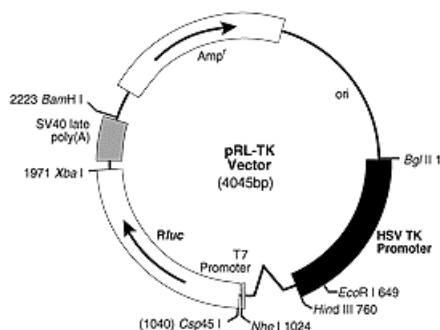


Figure 9. Circle map of the pRL-TK Vector. Additional description: ^, position of intron; *Rluc*, cDNA encoding the *Renilla* luciferase enzyme; *Amp^r*, gene conferring ampicillin resistance in *E. coli*; *ori*, origin of plasmid replication in *E. coli*. Arrows within the *Rluc* and *Amp^r* gene indicate the direction of transcription.

pRL-SV40

The pRL-SV40 Vector contains the SV40 early enhancer/promoter region that provides strong, constitutive expression of *Rluc* in a variety of cell types. The pRL-SV40 Vector also contains the SV40 origin of replication, which allows transient, episomal replication in cells expressing the SV40 large T antigen, such as COS-1 or COS-7 cells (10).

pRL-CMV

The pRL-CMV Vector contains the CMV** immediate-early enhancer/promoter region that provides strong, constitutive expression of *Rluc* in a variety of cell types. The promiscuous nature of the CMV enhancer/promoter has been demonstrated in transgenic mice, where its transcriptional activity was observed in 24 of the 28 murine tissues examined (11).

**The CMV Vector technology is the subject of U.S. Patent No. 5,168,062 assigned to The University of Iowa Research Foundation.

pRL-null

The pRL-null Vector lacks eukaryotic promoter and/or enhancer elements. An assortment of unique restriction enzyme sites located upstream of *Rluc* provide maximum flexibility in cloning desired promoter elements to drive *Renilla* luciferase expression.

The pRL-TK Vector depicted in [Figure 9](#) displays features common to all the pRL Vectors. Immediately downstream of the designated promoter in each pRL Vector is a chimeric intron that provides enhanced expression of *Renilla* luciferase. The intron is a composite of the 5'-donor splice site of human beta-globin intron 1, and the branch and 3'-acceptor splice site from an intron derived from the heavy chain variable region of an immunoglobulin gene (12). The sequences of the donor and acceptor splice sites, along with the branchpoint site, have been altered to match the consensus sequences for optimal splicing (13). Immediately upstream of *Rluc* is a T7 promoter sequence, allowing *in vitro* synthesis of *Renilla* luciferase transcripts. Downstream of *Rluc* is the SV40 late poly(A) sequence to provide efficient transcription termination and mRNA polyadenylation (14). The pRL Vectors contain a prokaryotic origin of replication and the beta-lactamase gene to allow selected plasmid propagation in *E. coli*.

Summary

In dual genetic reporter applications, changes in the activity of one reporter correlate to the effects of the specific experimental conditions on gene expression, while the constitutive activity of the second reporter provides an internal control by which experimental values can be normalized. Promega's new Dual-Luciferase™ Reporter Assay System integrates, in a single-tube format, the assays of two luciferase reporters having compatible chemistries, handling conditions, speed, sensitivity, linear range, and instrumentation requirements. The combination of the firefly and *Renilla* luciferase assays, in conjunction with the pRL Vectors, provides the ideal assay system for dual genetic reporter applications. The assay can be completed in 30 seconds using a standard manual luminometer. Moreover, the new Passive Lysis Buffer is specifically formulated to provide rapid and efficient passive lysis of cells cultured in multi-well plates, as well as optimal assay sensitivity and stability of the firefly and *Renilla* luciferases. The advantages of the Dual-Luciferase™ Reporter Assay System for *in vivo* reporter applications also apply to applications utilizing cell-free systems, such as *in vitro* transcription/translation reactions.

References

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Ordering Information

Product		Cat.#
Dual-Luciferase™ Reporter Assay System		E1910
Product	Size	Cat.#
pRL-TK Vector	20µg	E2241
pRL-SV40 Vector	20µg	E2231
pRL-CMV Vector	20µg	E2261
pRL-null Vector	20µg	E2271

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