

Dual-Luciferase® Reporter Assay System

Technical Manual No. 040

INSTRUCTIONS FOR USE OF PRODUCTS E1910 and E1960. *PLEASE DISCARD PREVIOUS VERSIONS.*
 All Technical Literature is Available on the Internet at www.promega.com

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I. Description

Genetic reporter systems are widely used to study eukaryotic gene expression and cellular physiology. Applications include the study of receptor activity, transcription factors, intracellular signaling, mRNA processing and protein folding. Dual reporters are commonly used to improve experimental accuracy. The term “dual reporter” refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. Typically, the “experimental” reporter is correlated with the effect of specific experimental conditions, while the activity of the co-transfected “control” reporter provides an internal control, which serves as the baseline response. Normalizing the activity of the experimental reporter to the activity of the internal control minimizes experimental variability caused by differences in cell

viability or transfection efficiency. Other sources of variability, such as differences in pipetting volumes, cell lysis efficiency and assay efficiency, can be effectively eliminated. Thus, dual reporter assays often allow more reliable interpretation of the experimental data by reducing extraneous influences.

Promega's Dual-Luciferase® Reporter (DLR™) Assay System^(a,b) provides an efficient means of performing dual reporter assays. In the DLR™ Assay, the activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*, also known as sea pansy) luciferases are measured sequentially from a single sample. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a “glow-type” luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is initiated by simultaneously adding Stop & Glo® Reagent to the same tube. The Stop & Glo® Reagent also produces a “glow-type” signal from the *Renilla* luciferase, which decays slowly over the course of the measurement. In the DLR™ Assay System, both reporters yield linear assays with subattomole sensitivities and no endogenous activity of either reporter in the experimental host cells. Furthermore, the integrated format of the DLR™ Assay provides rapid quantitation of both reporters either in transfected cells or in cell-free transcription/translation reactions.

Notice for Cat.# E1960: Sufficient lysis reagent (Passive Lysis Buffer, PLB) has been supplied to allow for addition of 20µl per well in 96 well plates. For applications requiring more lysis reagent (e.g., >100µl/well), additional PLB may be purchased separately (Cat.# E1941).

Promega's pRL family of *Renilla* luciferase control vectors (see Section III) has been designed for use with the DLR™ Assay Systems. The pRL Vectors^(c), which provide constitutive expression of *Renilla* luciferase, may be used in combination with any experimental firefly luciferase vector to co-transfect mammalian cells.

A. Dual-Luciferase® Reporter Assay Chemistry

Firefly and *Renilla* luciferases, because of their distinct evolutionary origins, have dissimilar enzyme structures and substrate requirements. These differences make it possible to selectively discriminate between their respective bioluminescent reactions. Thus, using the DLR™ Assay System, the luminescence from the firefly luciferase reaction (“experimental” reporter) may be quenched while simultaneously activating the luminescent reaction of *Renilla* luciferase (“control” reporter).

Firefly luciferase is a 61kDa monomeric protein that does not require post-translational processing for enzymatic activity (1,2). Thus, it functions as a genetic reporter immediately upon translation. Photon emission is achieved through oxidation of beetle luciferin in a reaction that requires ATP, Mg²⁺ and O₂ (Figure 1). Under conventional reaction conditions, the oxidation 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.

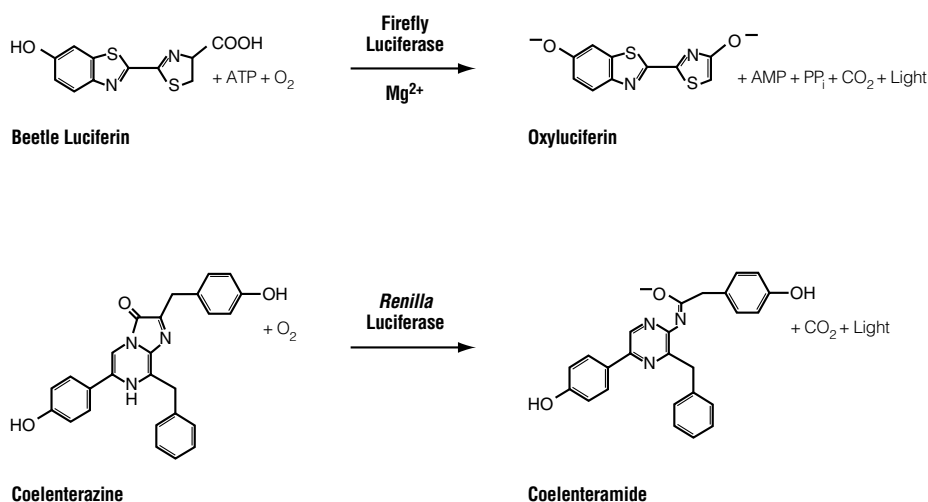


Figure 1. Bioluminescent reactions catalyzed by firefly and *Renilla* luciferases.

All of Promega's Luciferase Assay Reagents^(a) for quantitating firefly luciferase incorporate coenzyme A (CoA) to provide more favorable overall reaction kinetics (3). In the presence of CoA, the luciferase assay yields extended "glow-type" luminescence signals with significantly greater intensities (Figure 2) than those obtained from the conventional assay chemistry. The firefly luciferase assay is extremely sensitive and extends over a linear range covering at least seven orders of magnitude in enzyme concentration (Figure 3).

Renilla luciferase, a 36kDa monomeric protein, is composed of 3% carbohydrate when purified from its natural source, *Renilla reniformis* (4). However, like firefly luciferase, post-translational modification is not required for its 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). In 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). Similar to firefly luciferase, the luminescent reaction catalyzed by *Renilla* luciferase also provides extreme sensitivity and a linear range generally extending five orders of magnitude (Figure 3). Note that the effective range of the luminescent reactions may vary depending on the type of luminometer (e.g., 96 well vs. single sample) used.

An inherent property of coelenterazine is that it emits low-level autoluminescence in aqueous solutions. This drawback prevents more sensitive determinations at the lower end of enzyme concentration. Furthermore, some types of nonionic detergents commonly used to prepare cell lysates (e.g., Triton® X-100) greatly intensify coelenterazine autoluminescence. Promega's DLR™ Assay Systems include Passive Lysis Buffer, which is formulated to minimize the effect of lysate composition on coelenterazine autoluminescence. In addition, the DLR™ Assay Systems include two reconstituted assay reagents, Luciferase Assay Reagent II and Stop & Glo® Reagent, that combine to suppress coelenterazine autoluminescence. Thus, the DLR™ Assay System allows accurate, reproducible quantitation of very low levels of *Renilla* luciferase control activities expressed in prepared cell lysates.

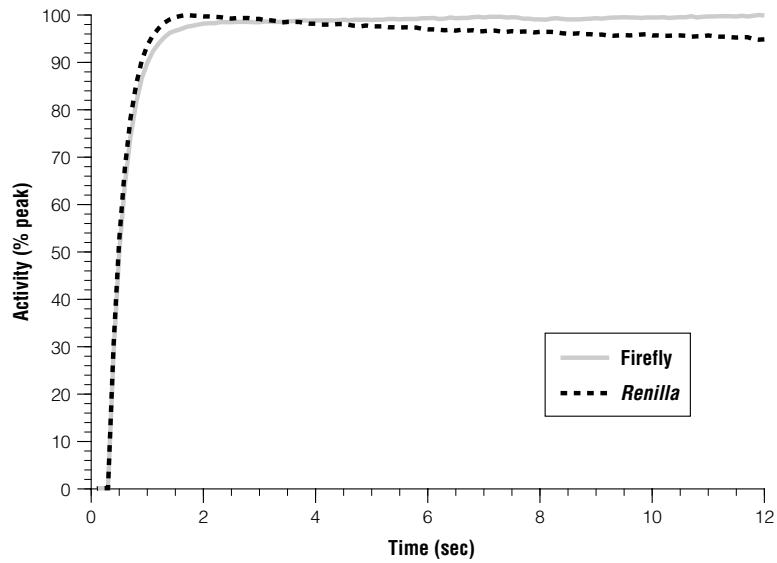


Figure 2. Luminescent signals generated in the Dual-Luciferase® Reporter 1000 Assay System by firefly and *Renilla* luciferases.

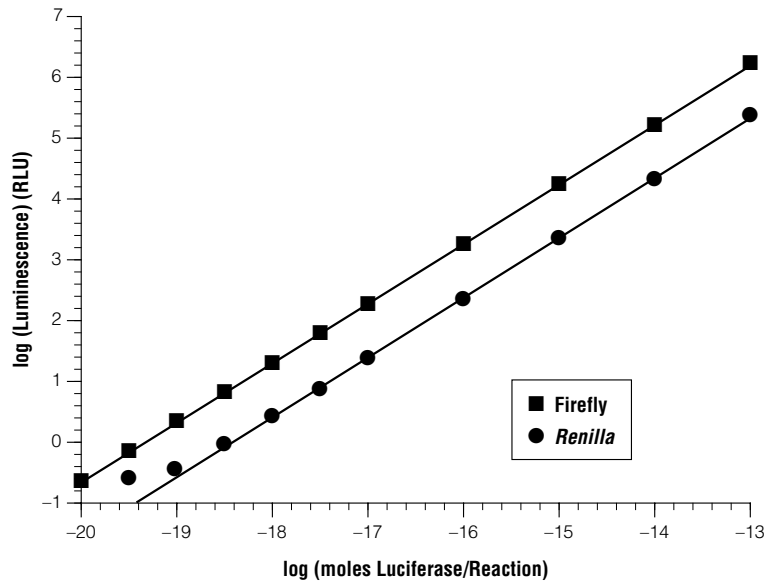


Figure 3. Comparison of the linear ranges of firefly and *Renilla* luciferases. The DLR™ Assay was performed with a mixture of purified firefly and *Renilla* luciferases prepared in PLB containing 1mg/ml gelatin. The instrumentation used was a Turner Designs Model 20e Luminometer. As shown in this graph with the DLR™ Assay System, the linear range of the firefly luciferase assay is seven orders of magnitude, providing detection sensitivity of ≤ 1 femtogram (approximately 10^{-20} mole) of experimental reporter enzyme. The *Renilla* luciferase assay has a linear range of greater than five orders of magnitude and allows for the detection of approximately 30 femtograms (approximately 3×10^{-19} moles) of control reporter enzyme.

B. Format of the Dual-Luciferase® Reporter Assay

Quantitation of luminescent signal from each of the luciferase reporter enzymes may be performed immediately following lysate preparation without the need for dividing samples or performing additional treatments. The firefly luciferase reporter assay is initiated by adding an aliquot of lysate to Luciferase Assay Reagent II. Quenching of firefly luciferase luminescence and concomitant activation of *Renilla* luciferase are accomplished by adding Stop & Glo® Reagent to the sample tube immediately after quantitation of the firefly luciferase reaction. The luminescent signal from the firefly reaction is quenched by at least a factor of 10⁵ (to ≤0.00001% residual light output) within 1 second following the addition of Stop & Glo® Reagent (Figure 4). Complete activation of *Renilla* luciferase is also achieved within this 1 second period. When using a manual luminometer, the time required to quantitate **both** luciferase reporter activities will be approximately 30 seconds. The procedure can be summarized as follows:

	Time
Step 1: Manually add prepared lysate to Luciferase Assay Reagent II predisposed into luminometer tubes; mix.	3 seconds
Step 2: Quantify firefly (experimental) luciferase activity.	12 seconds
Step 3: Add Stop & Glo® Reagent; mix.	3 seconds
Step 4: Quantitate <i>Renilla</i> (control) luciferase activity.	12 seconds
Total elapsed time for the DLR™ Assay	30 seconds

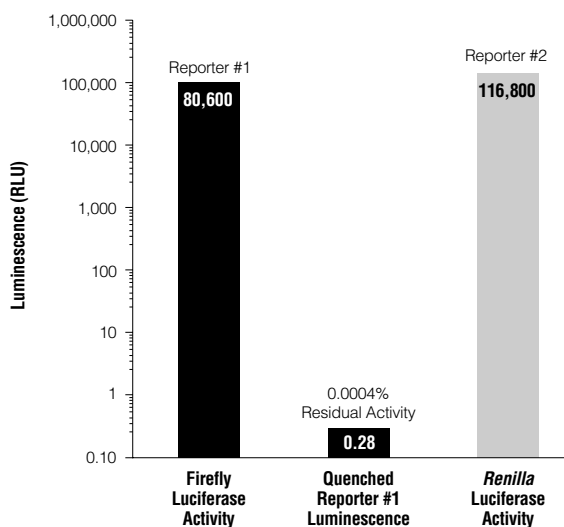


Figure 4. Measurement of luciferase activities before and after the addition of Stop & Glo® Reagent. The DLR™ Assay allows sequential measurement of firefly luciferase (Reporter 1), followed by *Renilla* luciferase activity (Reporter 2) on addition of Stop & Glo® Reagent to the reaction. Both reporter activities were quantitated within the same sample of lysate prepared from CHO cells co-transfected with pGL3 Control Vector^(d,e) (Cat.# E1741) and pRL-SV40 Vector^(c) (Cat.# E2231). To demonstrate the efficient quenching of Reporter 1 by Stop & Glo® Reagent, an equal volume of Stop & Glo® Buffer (which does not contain the substrate for *Renilla* luciferase) was added. Firefly luciferase luminescence was quenched by greater than 5 orders of magnitude.

C. Passive Lysis Buffer

Passive Lysis Buffer (PLB) is specifically formulated to promote rapid lysis of cultured mammalian cells without the need for scraping adherent cells and performing additional freeze/thaw cycles (active lysis). Furthermore, PLB prevents sample foaming, making it ideally suited for high-throughput applications in which arrays of treated cells are cultured in multi-well plates, processed into lysates and assayed using automated systems. Although PLB is formulated for passive lysis applications, its robust lytic performance is of equal benefit when harvesting adherent cells cultured in standard dishes using active lysis. Regardless of the preferred lysis method, the release of firefly and *Renilla* luciferase reporter enzymes into the cell lysate is both quantitative and reliable for cultured mammalian cells (Figure 5).

In addition to its lytic properties, PLB is designed to provide optimum performance and stability of the firefly and *Renilla* luciferase reporter enzymes. An important feature of PLB is that, unlike other cell lysis reagents, it elicits only minimal coelenterazine autoluminescence. Hence, PLB is the lytic reagent of choice when processing cells for quantitation of firefly and *Renilla* luciferase activities using the DLR™ Assay System. If desired, the protein content of cell lysates prepared with PLB may be readily quantitated using a variety of common chemical assay methods. Determination of protein content must be performed using adequate controls. Diluting lysates with either water or a buffer that is free of detergents or reducing agents is recommended in order to reduce the effects that Passive Lysis Buffer may have on background absorbance. A standard curve with BSA must be generated in parallel under the same buffer conditions.

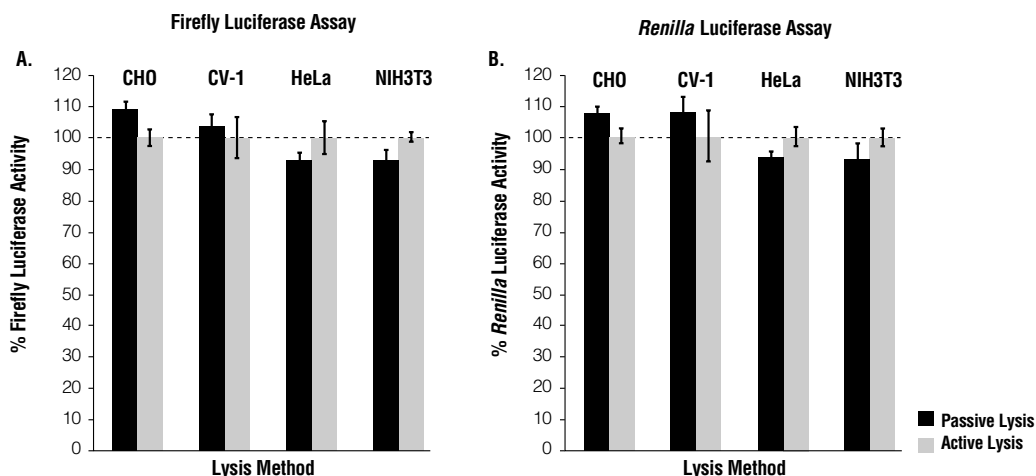


Figure 5. Comparison of firefly luciferase and *Renilla* luciferase reporter activities in cell lysates prepared with Passive Lysis Buffer using either the passive or active lysis procedure. Four different mammalian cell types were co-transfected with a firefly luciferase expression vector and a *Renilla* luciferase expression vector. Lysates were prepared by either exposing adherent cells to Passive Lysis Buffer for 15 minutes (passive lysis), or scraping adherent cells in the presence of Passive Lysis Buffer followed by one freeze/thaw cycle (active lysis). For comparative purposes, reporter activities were normalized to those obtained with the active lysis method for each cell type.

II. Product Components

Product	Size	Cat.#
Dual-Luciferase® Reporter Assay System	100 assays	E1910

Each system contains sufficient reagents to perform 100 standard Dual-Luciferase® Reporter Assays. Includes:

- 10ml Luciferase Assay Buffer II
- 1 vial Luciferase Assay Substrate (Lyophilized Product)
- 11ml Stop & Glo® Buffer
- 1 vial Stop & Glo® Substrate (Dried Product)
- 250µl Stop & Glo® Substrate Solvent
- 30ml Passive Lysis Buffer, 5X
- 1 Protocol

Product	Size	Cat.#
Dual-Luciferase® Reporter Assay System, 10-Pack	1,000 assays	E1960

Each system contains sufficient reagents to perform 1,000 standard Dual-Luciferase® Reporter Assays using 96 well luminometry plates. Includes:

- 10 × 10ml Luciferase Assay Buffer II
- 10 × 1 vial Luciferase Assay Substrate (Lyophilized Product)
- 10 × 11ml Stop & Glo® Buffer
- 10 × 1 vial Stop & Glo® Substrate (Dried Product)
- 10 × 250µl Stop & Glo® Substrate Solvent
- 30ml Passive Lysis Buffer, 5X
- 1 Protocol

Storage Conditions: Upon receipt of the Dual-Luciferase® Reporter Assay System, store at –20°C. Once the Luciferase Assay Substrate has been reconstituted, it should be divided into working aliquots and stored at –20°C for 1 month or at –70°C for up to 1 year. Reconstituted Stop & Glo® Substrate (Substrate + Substrate Solvent) may be stored at –20°C for 4 weeks or at –70°C for up to 1 year. Ideally, Stop & Glo® Reagent (Substrate + Substrate Solvent + Buffer) should be prepared fresh for each use. If necessary, this reagent may be stored at –20°C for 2 weeks.

III. The pRL Family of *Renilla* Luciferase Control Reporter Vectors

A. Description of pRL Vectors

The pRL family of *Renilla* luciferase control reporter vectors contain the cDNA encoding *Renilla* luciferase^(c) (*Rluc*) cloned from the anthozoan coelenterate *Renilla reniformis* (5), with some minor modifications for convenience as a genetic reporter (for more information, refer to the Technical Bulletin for each individual pRL Vector). The constitutive expression of *Rluc* is provided by one of several available promoter elements, as described below. In addition to the modified *Rluc* reporter gene, all pRL Vectors share the following features:

1. Immediately downstream of the designated promoter is a chimeric intron that provides enhanced expression of *Renilla* luciferase. The intron is a composite of the 5´-donor splice site of human β-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 (6). 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 (7).

Note: Regarding Cat.# E1960, for applications requiring more lysis reagent (e.g., >100µl/well), additional Passive Lysis Buffer may be purchased separately (Cat.# E1941).

Note: All Promega Technical Bulletins are available on the Internet at www.promega.com.

2. A T7 promoter is located immediately upstream of *Rluc*, allowing in vitro synthesis of *Renilla* luciferase transcripts using T7 RNA Polymerase.
3. The SV40 late poly(A) sequence is positioned downstream of *Rluc* to provide efficient transcription termination and mRNA polyadenylation (8).
4. A prokaryotic origin of replication and β -lactamase gene allows selected propagation of the pRL Vectors in *E. coli* host strains.

Currently, pRL Reporter Vectors are available in four promoter configurations:

pRL-SV40

The pRL-SV40 Vector contains the SV40 early enhancer/promoter region, which provides strong, constitutive expression of *Rluc* in a variety of cell types. The pRL-SV40 Vector contains the SV40 origin of replication, which allows transient, episomal replication in cells expressing the SV40 large T antigen, such as COS-1 and COS-7 cells (9). For more information on the pRL-SV40 Vector, request the *pRL-SV40 Vector Technical Bulletin*, #TB239.

pRL-CMV

The pRL-CMV Vector^(f) contains the CMV immediate-early enhancer/promoter region, which 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 (10). For more information on the pRL-CMV Vector, request the *pRL-CMV Vector Technical Bulletin*, #TB237.

pRL-TK

The pRL-TK Vector 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 (11,12). For more information on the pRL-TK Vector, request the *pRL-TK Vector Technical Bulletin*, #TB240.

pRL-null

The pRL-null Vector lacks eukaryotic promoter and/or enhancer elements upstream of *Rluc*. An array of unique restriction enzyme sites are located upstream of *Rluc*, providing maximum flexibility in cloning desired promoter elements to drive *Renilla* luciferase expression. For more information on the pRL-null Vector, request the *pRL-null Vector Technical Bulletin*, #TB238.

B. Important Considerations for Co-Transfection Experiments with pRL Vectors

The pRL family of co-reporter vectors may be used in combination with any experimental reporter vector to co-transfect mammalian cells. However, it is important to realize that *trans* effects between promoters on co-transfected plasmids can potentially affect reporter gene expression (13). Primarily, this is of concern when either the control or experimental reporter vector, or both, contain very strong promoter/enhancer elements. The occurrence and magnitude of such effects will depend on the combination and activities of the genetic regulatory elements present on the co-transfected vectors, the relative ratio of experimental vector to control vector introduced into the cells, and the cell type transfected.

As described in Section A, the pRL Vectors are available in a variety of promoter configurations. The HSV-thymidine kinase promoter (pRL-TK) is relatively weak and may be particularly useful in providing neutral constitutive expression of the *Renilla* luciferase control reporter. The early SV40 enhancer/promoter region (pRL-SV40) and the CMV immediate early enhancer/promoter region (pRL-CMV) typically provide high-level transcription and, therefore, may be less suitable for co-reporter applications involving experimental vectors with robust regulatory elements. In general, we recommend validating the performance of specific co-reporter combinations in the desired target cells.

To help ensure independent genetic expression between experimental and control reporter genes, we encourage users to perform preliminary co-transfection experiments to optimize both the **amount** of vector DNA and the **ratio** of co-reporter vectors added to the transfection mix. The extreme sensitivity of both firefly and *Renilla* luciferase assays, and the very large linear range of luminometers (typically 5–6 orders of magnitude), allows accurate measurement of even vastly different experimental and control luminescence values. Therefore, it is possible to add relatively small quantities of a pRL Vector to provide low-level, constitutive expression of *Renilla* luciferase control activity. Ratios of 10:1 to 50:1 (or greater) for experimental vector:co-reporter vector combinations are feasible and may aid greatly in suppressing the occurrence of *trans* effects between promoter elements. Alternatively, depending on the relative levels of reporter gene expression from the experimental and control vectors, it may be preferable to provide the firefly luciferase vector as the smaller component of the transfection mix.

IV. Instrument Considerations

A. Single-Sample Luminometers

Renilla and firefly luciferases both exhibit glow-type reaction kinetics; therefore, single-sample luminometers designed for low-throughput applications do not require reagent injectors to perform DLR™ Assays. Luminometers should be configured to measure light emission over a defined period, as opposed to measuring “flash” intensity or “peak” height. For the standard DLR™ Assay, we recommend programming luminometers to provide a 2-second preread delay, followed by a 10-second measurement period. However, depending on the type of instrument and the number of samples being processed, some users may prefer to shorten the period of premeasurement delay and/or the period of luminescence measurement. For convenience, it is preferable to equip the luminometer with a computer or an online printer for direct capture of data output, thus eliminating the need to pause between reporter assays to manually record the measured values. Some single-tube luminometers equipped with one or two reagent injectors may be difficult, or impossible, to reprogram to accommodate the “read-inject-read” format of the DLR™ Assay. In such instances, we recommend disabling the injector system and manually adding the reagent.

The Turner Designs Model TD-20/20 Luminometer, equipped with single or dual auto-injector systems (Cat.# E2351 or E2061) and printer, is ideally suited for low-throughput processing of DLR™ Assays. The TD-20/20 Luminometer is pre-programmed to perform injections and to complete sequential readings of both firefly and *Renilla* luciferase reporter activities with a single “Start” command. Furthermore, the instrument is programmed to provide the individual and normalized luciferase values, as well as statistical analyses of values measured within replicate groups.



Verify

... that your luminometer provides a diagnostic warning when the luminescence of a given sample exceeds the linear range of the photomultiplier tube.

B. Multi-Sample and Plate-Reading Luminometers

The most convenient method of performing large numbers of DLR™ Assays is to use a luminometer capable of processing multiple sample tubes, or by configuring assays in a 96 well array and using a plate-reading luminometer. For high-throughput applications, we recommend first dispensing the desired volume of each sample into the individual assay tubes or wells of the microplate. Dual-reporter assays are then performed according to the following steps: 1) inject Luciferase Assay Reagent II, 2) measure firefly luciferase activity, 3) inject Stop & Glo® Reagent and 4) measure *Renilla* luciferase activity. Therefore, multi-sample and plate-reading luminometers should be equipped with at least two reagent injectors to perform the DLR™ Assay. Users of high-throughput instruments will likely be able to perform DLR™ Assays using elapsed premeasurement and measurement times that are significantly shorter than those prescribed in the standard assay protocol.

Note: It is common for the luminescence intensity of luciferase-mediated reactions to exceed the linear range of a luminometer. Verify that your luminometer provides a diagnostic warning when the luminescence of a given sample exceeds the linear range of the photomultiplier tube. If it does not, it is important to establish the luminometer's linear range of detection prior to performing luciferase reporter assays. Purified luciferase (e.g., QuantiLum™ Recombinant Luciferase; Cat.# E1701), or luciferase expressed in cell lysates, may be used to determine the working range of a particular luminometer. Perform serial dilutions of the luciferase sample in 1X PLB (refer to Section V.A) containing 1mg/ml gelatin. The addition of exogenous protein is necessary to ensure stability of the luciferase enzyme at extremely dilute concentrations.

C. Scintillation Counters

In general, we do not recommend the use of scintillation counters for quantitating firefly and *Renilla* luciferase activities using the integrated DLR™ Assay chemistry. Scintillation counters are not equipped with auto-injection devices; therefore, samples assayed using the Dual-Luciferase® format must be processed manually. Since the luminescent signal generated by both luciferases decays slowly over the course of the reaction period (Figure 2), it is necessary to operate the scintillation counter in manual mode and to initiate each reaction just prior to measurement. This is especially important for the *Renilla* luciferase reaction, which decays more rapidly than the firefly luciferase reaction. As a result of this decay, it is also important to control the elapsed time between initiating the reaction and taking a measurement.

If a scintillation counter is used to measure firefly and *Renilla* luciferase activities, configure the instrument so that all channels are open, and the coincidence circuit is turned off. This is usually achieved through an option of the programming menu or by a switch within the instrument. If the circuit cannot be turned off, a linear relationship between luciferase concentration and cpm can still be produced by calculating the square root of measured counts per minute (cpm) minus background cpm (i.e., $[\text{sample} - \text{background}]^{1/2}$). See Section VI.E for a discussion of performing background measurements.

V. Preparation of Cell Lysates Using Passive Lysis Buffer

Two procedures are described for the preparation of cell lysates using PLB. The first is recommended for the passive lysis of cells in multi-well plates. The second is intended for those who are harvesting cells grown in culture dishes and prefer to expedite lysate preparation by scraping the adherent cells. In both procedures, the firefly and *Renilla* luciferases contained in the cell lysates prepared with PLB are stable for at least 6 hours at room temperature (22°C) and up to 16 hours on ice. Freezing at -20°C is suitable for short-term storage (up to 1 month) of prepared lysates; however, we recommend long-term storage at -70°C. Subjecting cell lysates to more than 2–3 freeze/thaw cycles may result in gradual loss of luciferase reporter enzyme activities.

Materials to Be Supplied by the User

(Solution composition is provided in Section VIII.A.)

- phosphate buffered saline (PBS)

A. Passive Lysis Buffer Preparation

PLB is supplied as a 5X concentrate. Prepare a sufficient quantity of the 1X working concentration by adding 1 volume of 5X Passive Lysis Buffer to 4 volumes of distilled water and mixing well. The diluted (1X) PLB may be stored at 4°C for up to one month; however, it is recommended that PLB be prepared fresh in volumes as required for experimental use. The 5X PLB should be stored at -20°C.

B. Passive Lysis of Cells Cultured in Multi-Well Plates

- Determine transfection parameters (i.e., plated cell density and subsequent incubation time) such that cells are no more than 95% confluent at the desired time of lysate preparation. Remove the growth medium from the cultured cells, and **gently** apply a sufficient volume of phosphate buffered saline (PBS) to wash the surface of the culture vessel. Swirl the vessel briefly to remove detached cells and residual growth medium. Completely remove the rinse solution before applying PLB reagent.
- Dispense into each culture well the minimum volume of 1X PLB that is required to completely cover the cell monolayer. The recommended **minimum** volumes of PLB to be added per well are as follows:

Multi-Well Plate	1X PLB
6 well culture plate	500µl
12 well culture plate	250µl
24 well culture plate	100µl
48 well culture plate	65µl
96 well culture plate	20µl

- Place the culture plates on a rocking platform or orbital shaker with gentle rocking/shaking to ensure complete and even coverage of the cell monolayer with 1X PLB. Rock the culture plates at room temperature for 15 minutes.
- Transfer the lysate to a tube or vial for further handling and storage. Alternatively, reporter assays may be performed directly in the wells of the culture plate. In general, it is unnecessary to clear lysates of residual cell debris prior to performing the DLR™ Assay. However, if subsequent protein determinations are to be performed, we recommend clearing the lysate

Note: An *Experienced User's Protocol* can be found at the end of this Technical Manual.



Only Use

Passive Lysis Buffer with the DLR™ Assay System since PLB is specially formulated to minimize background autoluminescence.



Some Cell

Types may exhibit greater inherent resistance to lysis, and optimizing the treatment conditions may be required.

samples by centrifugation for 30 seconds at top speed in a refrigerated microcentrifuge. Transfer cleared lysates to a fresh tube prior to reporter enzyme analyses.

Notes:

1. Cultures that are overgrown are often more resistant to complete lysis and typically require an increased volume of PLB and/or an extended treatment period to ensure complete passive lysis. Firefly and *Renilla* luciferases are stable in cell lysates prepared with PLB (14); therefore, extending the period of passive lysis treatment will not compromise reporter activities.
2. Microscopic inspection of different cell types treated for passive lysis may reveal seemingly different lysis results. Treatment of many types of cultured cells with PLB produces complete dissolution of their structure within a 15-minute period. However, PLB treatment of some cell types may result in discernible cell silhouettes on the surface of the culture well, or large accumulations of floating debris. Despite the appearance of such cell remnants, we typically find complete solubilization of both luciferase reporter enzymes within a 15-minute treatment period (See Figure 5). However, some types of cultured cells may exhibit greater inherent resistance to lysis, and optimizing the treatment conditions may be required.

C. Active Lysis of Cells by Scraping

1. Remove growth medium from the cultured cells, and **gently** apply a sufficient volume of PBS to rinse the bottom of the culture vessel. Swirl the vessel briefly to remove detached cells and residual growth medium. Take care to completely remove the rinse solution before applying the 1X PLB.
2. Homogenous lysates may be rapidly prepared by manually scraping the cells from culture dishes in the presence of 1X PLB. Recommended **minimum** volumes of PLB to be added per culture dish are listed below.

Cell Culture Plate	1X PLB
100 × 20mm culture dish	1.00ml
60 × 15mm culture dish	400µl
35 × 12mm culture dish	200µl
6 well culture plate	250µl
12 well culture plate	100µl

3. Cells may be harvested immediately following the addition of PLB by scraping vigorously with a disposable plastic cell lifter or a rubber policeman. Tilt the plate, and scrape the lysate down to the lower edge. Pipet the accumulated lysate several times to obtain a homogenous suspension. If the scraper is used to prepare more than one sample, thoroughly clean the scraper between uses.
4. Transfer the lysate into a tube or vial for further handling and storage. Subject the cell lysate to 1 or 2 freeze/thaw cycles to accomplish complete lysis of cells. Generally, it is unnecessary to clear lysates of residual cell debris prior to performing the DLR™ Assay. However, if subsequent protein determinations are to be performed, we recommend clearing the lysate samples by centrifugation for 30 seconds in a refrigerated microcentrifuge. Transfer the cleared lysates to a fresh tube prior to reporter enzyme analyses.

Note: An *Experienced User's Protocol* can be found at the end of this Technical Manual.

VI. Dual-Luciferase® Reporter Assay Protocol

Materials to Be Supplied by the User

- luminometer
- siliconized polypropylene tube or small glass vial

A. Preparation of Luciferase Assay Reagent II

Prepare Luciferase Assay Reagent II (LAR II) by resuspending the provided lyophilized Luciferase Assay Substrate in 10ml of the supplied Luciferase Assay Buffer II. Once the substrates and buffer have been mixed, write "LAR II" on the existing vial label for easy identification. LAR II is stable for one month at -20°C or for one year when stored at -70°C .

Notes:

1. Repeated freezing/thawing of this reagent may decrease assay performance. We recommend that LAR II be dispensed into aliquots for each experimental use (e.g., 1ml aliquots will each provide 10 assays).
2. The components of LAR II are heat-labile. Frozen aliquots of this reagent should be thawed in a water bath at room temperature.
3. The process of thawing generates both density and composition gradients within LAR II. Mix the thawed reagent prior to use by inverting the vial several times or by gentle vortexing.

B. Preparation of Stop & Glo® Reagent

1. Transfer 200 μl of Stop & Glo® Substrate Solvent into the amber glass vial containing dried Stop & Glo® Substrate (transfer only 200 μl of Stop & Glo® Substrate Solvent from the 250 μl total volume provided). Replace the butyl stopper, and gently vortex for 10 seconds. If the substrate solution splashes onto the stopper, place bottle in a 50ml tube and centrifuge at $30 \times g$ for 2 minutes to return the substrate to the bottom of the bottle. The reconstituted Stop & Glo® Substrate should be bright yellow. Write "50X" on the existing vial label to indicate the concentration of the stock solution. Reconstituted Stop & Glo® Substrate may be stored for 4 weeks at -20°C or up to 1 year at -70°C .
2. Stop & Glo® Reagent (Substrate + Substrate Solvent + Buffer) is best when prepared fresh. It is stable for 12 hours at room temperature. If necessary, Stop & Glo® Reagent may be stored at -20°C for 2 weeks or up to 1 month when stored frozen at -70°C . It may be thawed at room temperature up to two times without appreciable activity loss.

Prepare an adequate volume to perform the desired number of DLR™ Assays (100 μl reagent per assay). Add 1 volume of reconstituted 50X Stop & Glo® Substrate (prepared in Step 1) to 50 volumes of Stop & Glo® Buffer in a glass or siliconized polypropylene tube.

Example 1 (10 assays)

Add 20 μl of 50X Stop & Glo® Substrate to 1ml of Stop & Glo® Buffer contained in either a glass vial or siliconized polypropylene tube. This will prepare sufficient Stop & Glo® Reagent to perform 10 assays.



Do Not

substitute Promega's Luciferase Assay Reagent (included in Cat.#s E1500, E1501, E4030, E4530, E4550 & E1483) for LAR II. It is not designed for use with the DLR™ Assay System.



Stop & Glo®

Substrate Solvent may be a mild irritant; therefore, appropriate precautions should be taken to prevent skin and eye contact. Careful pipetting of the solvent is necessary because of its volatile nature.



Prior To

Beginning this protocol, verify that the LAR II and the Stop & Glo® Reagent have been prepared fresh or have been thawed in a room temperature water bath.



Do Not

...vortex at Step 3.



Priming Assay

Reagent through an empty injector system prevents dilution and contamination of the primed reagent.

Example 2 (100 assays)

Transfer 10ml Stop & Glo® Buffer into a glass vial or siliconized polypropylene tube. Add the entire 200µl volume of 50X Stop & Glo® Substrate to the 10ml Stop & Glo® Buffer. Rinse the substrate vial with 1ml from the 10ml Stop & Glo® Reagent to assure complete transfer of substrate solution. Add this 1ml rinse back to the vial of Stop & Glo® Reagent. This will prepare sufficient Stop & Glo® Reagent to perform 100 DLR™ Assays.

Note: Reagents that have been prepared and stored frozen should be thawed in a room temperature water bath. Always mix the reagent prior to use because thawing generates density and composition gradients.

C. Standard Protocol

The assays for firefly luciferase activity and *Renilla* luciferase activity are performed sequentially using one reaction tube. The following protocol is designed for use with a manual luminometer, or a luminometer fitted with one reagent injector (Figure 6).

Note: In some instances, it may be desirable to measure **only** *Renilla* luciferase reporter activity in the lysates of pRL Vector-transfected cells. If the DLR™ Assay System is used to measure only *Renilla* luciferase activity, it is still necessary to combine 100µl of both LAR II and Stop & Glo® Reagent with 20µl cell lysate to achieve optimal *Renilla* luciferase assay conditions.

1. Predispense 100µl of LAR II into luminometer tubes required to complete the desired number of DLR™ Assays.
2. Program the luminometer to perform a 2-second premeasurement delay, followed by a 10-second measurement period for each reporter assay.
3. Carefully transfer up to 20µl of cell lysate into the luminometer tube containing LAR II; mix by pipetting 2 or 3 times. Place the tube in the luminometer and initiate reading.

Note: We do not recommend vortexing the solution at this step. Vortexing may coat the sides of the tube with a microfilm of luminescent solution, which can escape mixing with the subsequently added volume of Stop & Glo® Reagent. This is of particular concern if Stop & Glo® Reagent is delivered into the tube by automatic injection.

4. If the luminometer is not online with a printer or computer, record the firefly luciferase activity measurement.
5. If available, use a reagent injector to dispense 100µl of Stop & Glo® Reagent. If using a manual luminometer, remove the sample tube from the luminometer; add 100µl of Stop & Glo® Reagent; and vortex briefly to mix. Replace the sample in the luminometer, and initiate reading.

Note: It is possible to prime auto-injector systems with little or no loss of DLR™ Assay reagents. Before priming injectors with LAR II or Stop & Glo® assay reagents, we recommend first purging all storage liquid (i.e., deionized water or ethanol wash solution; see Section VI.D, Step 2) from the injector system. Priming assay reagent through an empty injector system prevents dilution and contamination of the primed reagent. Thus, the volume of primed reagent may be recovered and returned to the reservoir of bulk reagent.

6. If the luminometer is not online with a printer or computer, record the *Renilla* luciferase activity measurement.
7. Discard the reaction tube, and proceed to the next DLR™ Assay.

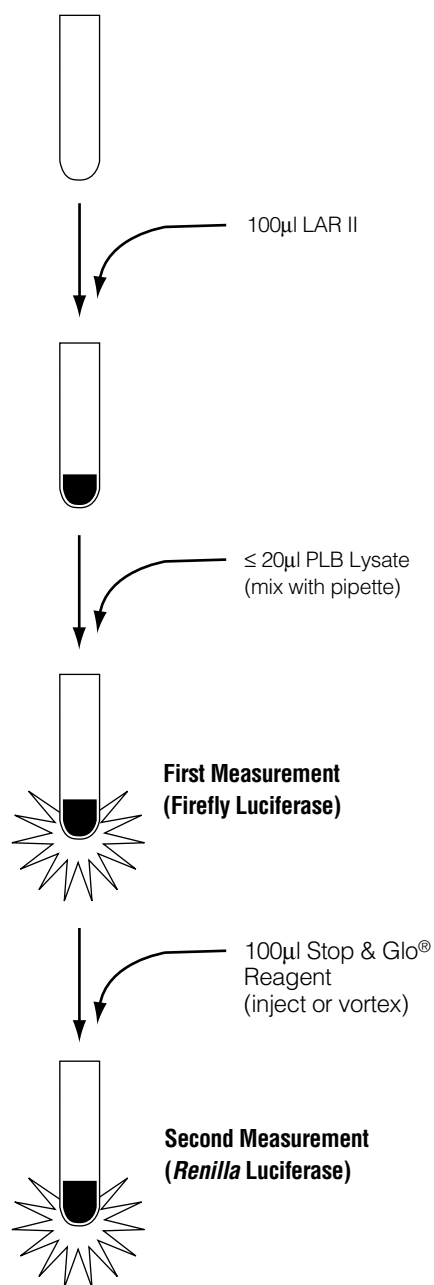


Figure 6. Format of the DLR™ Assay using a manual luminometer or a luminometer equipped with one reagent injector. If the instrument is equipped with two injectors, it may be preferable to predispense the lysate into luminometer tubes, followed by sequential auto-injection of the LAR II and Stop & Glo® Reagents.



Proper

Cleaning of an injector system exposed to Stop & Glo® Reagent is essential if the device is to be later used to perform firefly luciferase assays by auto-injecting LAR II.

D. Important Considerations for Cleaning Reagent Injectors

One of the luciferase-quenching components in Stop & Glo® Reagent has a moderate affinity for plastic materials. This compound exhibits a reversible association with the interior surfaces of plastic tubing and pump bodies commonly used in the construction of auto-injector systems. Injector plumbing that has not been properly cleaned following contact with Stop & Glo® Reagent will leach trace quantities of quench reagent into solutions that are subsequently passed through the injector system. In such cases, even very small quantities of contaminating quench reagent cause significant inhibition of firefly luciferase reporter activity. Hence, proper cleaning of an injector system exposed to Stop & Glo® Reagent is essential if the device is to be later used to perform firefly luciferase assays by auto-injecting LAR II. It is recommended that a particular injector be dedicated to each of the two reagents. Whether using one or two injectors, it is important to use the following wash protocol to ensure clean lines and a well-maintained instrument.

General Injector Wash Protocol

1. Purge Stop & Glo® Reagent from the injector lines by repeated priming/washing with a volume of deionized water equivalent to 3 pump void volumes.
2. Prepare 70% ethanol as wash reagent. Prime the system with at least 5ml 70% ethanol to completely replace the void volume and rinse the injector plumbing. It is preferable to allow the injector to soak in this wash solution for 30 minutes prior to rinsing with deionized water.

Note: The design and materials used in the construction of injector systems varies greatly, and some pumps may require longer than a 30-minute soak in the wash reagent to attain complete surface cleaning. Luminometers equipped with Teflon® tubing are not a concern, but other tubing such as Tygon® will require an extended soak time of 12–16 hours (overnight) to ensure complete removal of the Stop & Glo® Reagent from the injector system.

3. Rinse with a volume of deionized water equivalent to at least 3 pump void volumes to thoroughly remove all traces of ethanol.

Wash Protocol for the Injectors in the Turner Designs TD-20/20 Luminometer

The TD-20/20 Luminometer requires at least 5 priming cycles to achieve 100% displacement of the solution contained within the injector plumbing. Trace contamination of Stop & Glo® Reagent may be removed from the TD-20/20 Luminometer injector system as follows:

1. Purge Stop & Glo® Reagent from the injector by performing 10 priming cycles with deionized water.
2. Perform at least 10 priming cycles with 70% ethanol and allow tubing to soak in this wash solution for 30 minutes.
3. Perform at least 10 priming cycles with deionized water to remove all traces of ethanol.

E. Determination of Assay Backgrounds

The expression of a luciferase reporter is quantitated as the luminescence produced above background levels. In most cases, because the background is exceptionally low, luciferase activity is directly proportional to total luminescence. However, when measuring very small amounts of luciferase, it is important to subtract the background signal from the measurement of total luminescence. The following sections describe how to determine background signals for firefly and *Renilla* luciferases, respectively.

Firefly Luciferase

With rare exceptions, all background in measurements of firefly luciferase arises from the instrumentation or the sample tubes. Background in sample tubes may result from static electricity or from phosphorescence. In particular, polystyrene tubes are capable of accumulating significant static buildup that may contribute to persistent, elevated levels of background luminescence. Handling and storage of tubes should be done to minimize static buildup, and samples should be handled away from sunlight or very bright lights before making luminescence measurements.

The electronic design of a given luminometer can greatly affect its measurable level of background signal; many luminometers do not read "0" in the absence of a luminescent sample. To determine the background signal contributed by the instrument and sample tube:

1. Use Passive Lysis Buffer to prepare a lysate of nontransfected control (NTC) cells.
2. Add 20 μ l of NTC lysate to 100 μ l of LAR II.
3. Measure apparent luminescence activity.

Because the lysates of mammalian cells do not express endogenous luminescence activity, the low apparent luminescence in NTC lysates is the background due to the instrument and possibly the sample tubes. Be aware that the relative noise in background signals is often quite high. Therefore, 5–10 readings should be performed, and the mean of these readings should be used to obtain a statistically significant value for instrument and sample tube background.

Renilla Luciferase

Background luminescence in the measurement of *Renilla* luciferase activity can arise from three possible sources:

1. Instrument and sample tube background luminescence: This is the same as for firefly luciferase.
2. Autoluminescence of coelenterazine: This is caused by nonenzymatic oxidation of the coelenterazine in solution. Although the level of autoluminescence is dependent on solution composition, lysates prepared with PLB generally yield a low and constant luminescence level.
3. Residual luminescence from the firefly luciferase reaction: This can occur from a small amount of residual luminescence remaining from the firefly luciferase assay in the Dual-Luciferase[®] measurement. However, since the firefly luciferase reaction is quenched greater than 100,000-fold, this residual luminescence is only significant if the *Renilla* luciferase luminescent reaction is 1,000-fold less than the intensity of the first firefly luciferase luminescent reaction.

For questions not addressed here, please contact your local Promega Branch Office or distributor. Contact information available at: www.promega.com.

E-mail: techserv@promega.com



Five To Ten

background readings should be performed, and the mean reading used to obtain a statistically significant value for instrument and sample tube background.

The background luminescence contributed by (1) and (2) above is constant and can be subtracted from all measurements of *Renilla* luciferase. Because the background from (3) is variable, depending on the expression of firefly luciferase, it may be important to verify that the maximum expected level of firefly luciferase activity does not yield significant residual luminescence that may affect the accurate measurement of *Renilla* luciferase. Such a circumstance may arise as a result of incomplete mixing of the Stop & Glo[®] Reagent with the sample LAR II. In addition, if the first injection of LAR II coats the walls of the tube but the second injection with the Stop & Glo[®] Reagent covers less surface area, inadequate quenching will be obtained.

To determine the background contributed by the instrument, sample tube and coelenterazine autoluminescence, do the following:

1. Use Passive Lysis Buffer to prepare a lysate of nontransfected control (NTC) cells.
2. Add 20µl of the NTC cell lysate to a luminometer tube containing 100µl of Luciferase Assay Reagent II.
3. Add 100µl of Stop & Glo[®] Reagent to the sample tube.
4. Measure background.

To determine the background from residual firefly luciferase luminescence, do the following:

1. Use Passive Lysis Buffer to prepare a lysate of cells expressing high levels of firefly luciferase.
2. Add 20µl of the cell lysate to a luminometer tube containing 100µl of Luciferase Assay Reagent II.
3. Measure firefly luciferase luminescence.
4. Add 100µl of Stop & Glo[®] Reagent.
5. Measure apparent luminescence.
6. Subtract background contributed from coelenterazine autoluminescence plus instrument background (as determined above).

For a very strong firefly luciferase reaction, the background-subtracted value of quenched luminescence measured in Step 6 should be 100,000-fold less than the value of firefly luciferase luminescence measured in Step 3. In most instances the value for firefly luminescence will not be 100,000-fold greater than the background value alone. Therefore, it is unlikely that significant residual firefly luminescence signal will be detectable above the background measured in Step 5.

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VIII. Appendix

A. Composition of Buffers and Solutions

PBS buffer, 10X (per liter)

11.5g	Na ₂ HPO ₄
2g	KH ₂ PO ₄
80g	NaCl
2g	KCl

Dissolve in 1 liter of sterile, deionized water.
The pH of 1X PBS will be 7.4.

B. Related Products

Product	Size	Cat.#
Dual-Luciferase® Reporter 1000 Assay System(a,b)	1,000 assays	E1980
QuantiLum™ Recombinant Luciferase(e)	1mg	E1701
	5mg	E1702
pRL-SV40 Vector	20µg	E2231
pRL-TK Vector	20µg	E2241
pRL-CMV Vector	20µg	E2261
pRL-null Vector	20µg	E2271
Passive Lysis Buffer, 5X	30ml	E1941

Please call Promega Technical Services to inquire about the availability of new promoter variations within the pRL family of co-reporter vectors. To inquire about the availability of bulk packaging and pricing for individual pRL Vectors, please contact Promega.

Product	Cat.#
Turner Designs Model TD-20/20 Luminometer	E2041
Turner Designs Model TD-20/20 Luminometer with Printer	E2051
Turner Designs Model TD-20/20 Luminometer with Single Auto Injector	E2351
Turner Designs Model TD-20/20 Luminometer with Dual Auto Injector	E2361
Turner Designs Model TD-20/20 Luminometer with Printer and Dual Auto Injector	E2061

Product	Size	Cat.#
<i>Renilla</i> Luciferase Assay System(g,h)	100 assays	E2810
	1,000 assays	E2820
phRL-null Vector(h,i,k)	20µg	E6231
phRL-TK Vector(h,i,k)	20µg	E6241
phRL-TK(Int ⁻) Vector(h,i,k)	20µg	E6251
phRL-SV40 Vector(h,i,k)	20µg	E6261
phRL-CMV Vector(h,i,j,k)	20µg	E6271
phRG-B Vector(h,i,k)	20µg	E6281
phRG-TK Vector(h,i,k)	20µg	E6291

(a) U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289, 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.

(b) U.S. Pat. No. 5,744,320 has been issued to Promega Corporation for quenching reagents and assays for enzyme-mediated luminescence.

(c) The cDNA encoding luciferase from *Renilla reniformis* is covered by U.S. Pat. No. 5,292,658 assigned to the University of Georgia Research Foundation, Inc., and sublicensed from SeaLite Sciences, Inc., Norcross, GA. The pRL family of *Renilla* luciferase cDNA vectors is for research use only. Commercial manufacture would require a license from SeaLite Sciences, Inc.

(d) U.S. Pat. No. 5,670,356 has been issued to Promega Corporation for a modified luciferase technology.

(e) The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

(f) Covered under U.S. Pat. No. 5,168,062 assigned to the University of Iowa Research Foundation.

(g) Patent Pending.

(h) Certain applications of this product may require licenses from others.

(i) Licensed under U.S. Pat. Nos. 5,292,658, 5,418,155 and other patents.

(j) The CMV promoter and its use are covered under U.S. Pat. Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation, Iowa City, Iowa, and licensed FOR RESEARCH USE ONLY. Commercial users must obtain a license to these patents directly from the University of Iowa Research Foundation.

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Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.

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Notes

Dual-Luciferase® Reporter Assay System: *Experienced User's Protocol*

This quick protocol is intended as an easy-to-follow reminder for experienced users. Please follow the complete protocol (Sections V.A through VI.C) the first time you use the Dual-Luciferase® Reporter Assay System.

<p>Before You Begin</p>	<ol style="list-style-type: none"> 1. Select an appropriate <i>Renilla</i> Luciferase control co-reporter vector (Section III). 2. Prepare reagents as directed in Sections V.A, VI.A and VI.B. Briefly: <ol style="list-style-type: none"> a. Add 1 volume of 5X Passive Lysis Buffer to 4 volumes of distilled water; mix well. b. Add 10ml of the supplied Luciferase Assay Buffer II to the lyophilized Luciferase Assay Substrate. Mix to dissolve. c. Add 200µl of Stop & Glo® Substrate Solvent to the dried Stop & Glo® Substrate. Gently vortex for 10 seconds to solubilize the substrate. (This is a 50X solution). d. To prepare 1X Stop & Glo® Reagent, add an adequate volume of 50X Stop & Glo® Substrate to 50 volumes Stop & Glo® Buffer in a glass or siliconized polypropylene tube. For example, add 20µl Stop & Glo® Substrate to 1ml Stop & Glo® Buffer to prepare sufficient Stop & Glo® Reagent for 10 assays.
<p>Passive or Active Lysis of Cells (Section V.B and V.C)</p>	<ol style="list-style-type: none"> 1. Remove the growth medium from the cultured cells, and gently apply PBS to wash the surface of the culture vessel. Swirl the vessel briefly. Completely remove the rinse solution. 2. Dispense into each culture vessel the recommended minimum volume of 1X PLB for the culture vessel. See Sections V.B and V.C for recommended volumes for passive and active lysis, respectively. 3. For passive lysis, place the culture plates on a rocking platform or orbital shaker with gentle rocking/shaking to ensure complete and even coverage of the cell monolayer with 1X PLB. Rock the culture plates at 25°C for 15 minutes. Transfer the lysate to a tube or vial. For active lysis, harvest cells immediately following the addition of 1X PLB by scraping vigorously with a disposable plastic cell lifter or a rubber policeman. Tilt the plate, and scrape the lysate down to the lower edge. Pipet the accumulated lysate several times. Transfer the lysate to a tube or vial for handling and storage. Subject the cell lysate to 1 or 2 freeze-thaw cycles to accomplish complete lysis of cells.
<p>Protocol for Use with Manual or Single-Injector Luminometers (Section VI.C)</p>	<ol style="list-style-type: none"> 1. Predisperse 100µl of LAR II into luminometer tubes. 2. Program the luminometer to perform a 2-second premeasurement delay, followed by a 10-second measurement period for each reporter assay. 3. Carefully transfer up to 20µl of cell lysate to the luminometer tube containing LAR II; mix by pipetting 2–3 times. Place the tube in the luminometer. Initiate reading. Record the firefly luciferase activity measurement. 4. Dispense 100µl of Stop & Glo® Reagent. If using a manual luminometer, remove the sample tube from the luminometer; add 100µl of Stop & Glo® Reagent; and vortex briefly to mix. Replace the sample in the luminometer, and initiate reading. Record the <i>Renilla</i> luciferase activity measurement. 5. Discard the reaction tube, and proceed to the next DLR™ Assay.