IMPORTANT NOTICE

Promega is pleased to announce two improvements to the Dual-Luciferase® Reporter Assay Systems, Cat.# E1910, E1960 and E1980. The Stop & Glo® Substrate is now supplied in a liquid format for added convenience. The new liquid Stop & Glo® Substrate eliminates the substrate reconstitution step formerly required of these products. Consequently, the Stop & Glo® Substrate Solvent is no longer provided as a separate component in the DLR™ kits. In addition, the Stop & Glo® Buffer has been reformulated to reduce enzyme-independent luminescence in the *Renilla* luciferase reaction, resulting in increased sensitivity, and to remove an animal-derived component. In the new and improved kits, only the Stop & Glo® Substrate and Buffer have changed; all other components remain the same.

To ensure the high quality and performance of the new DLR™ Systems, we performed extensive functional and stability testing on the new Stop & Glo® Substrate. Stability testing supports long-term storage of the liquid substrate formulation. Promega's performance claims and product consistency for the DLR™ Systems have not changed. Thus, you can continue to expect optimal performance from the DLR™ Systems.

We value you as a customer and strive to continue to improve our products and services for you. If you need additional information or wish to request an information packet, please contact Promega's Technical Services at 800-356-9526 (608-274-4330 outside the US) or via email at techserv@promega.com

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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
Please visit the web site to verify that you are using the most current version of this Technical Manual.

I. Description 1
- A. Dual-Luciferase® Reporter Assay Chemistry 2
- B. Format of the Dual-Luciferase® Reporter Assay 5
- C. Passive Lysis Buffer 6

II. Product Components 7

III. The phRL and pRL Families of *Renilla* Luciferase Reporter Vectors 7
- A. Description of phRL and pRL Vectors 7
- B. Important Considerations for Co-Transfection Experiments 8

IV. Instrument Considerations 8
- A. Single-Sample Luminometers 8
- B. Multi-Sample and Plate-Reading Luminometers 9
- C. Scintillation Counters 9

V. Preparation of Cell Lysates Using Passive Lysis Buffer 10
- A. Passive Lysis Buffer Preparation 10
- B. Passive Lysis of Cells Cultured in Multiwell Plates 10
- C. Active Lysis of Cells by Scraping 11

VI. Dual-Luciferase® Reporter Assay Protocol 12
- A. Preparation of Luciferase Assay Reagent II 12
- B. Preparation of Stop & Glo® Reagent 12
- C. Standard Protocol 13
- D. Important Considerations for Cleaning Reagent Injectors 13
- E. Determination of Assay Backgrounds 14

VII. References 18

VIII. Appendix 18
- A. Composition of Buffers and Solutions 18
- B. Related Products 18

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 that 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

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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,c] 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 stabilized 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 stabilized 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 has three series of firefly and *Renilla* luciferase vectors, pGL3[d,e], phRL[c,f,g,h] and pRL[g], designed for use with the DLR™ Assay Systems. These vectors may be used to co-transfect mammalian cells with any experimental and control reporter genes.

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 may be quenched while simultaneously activating the luminescent reaction of *Renilla* luciferase.

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, Mg^{2+} and O_2 (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.

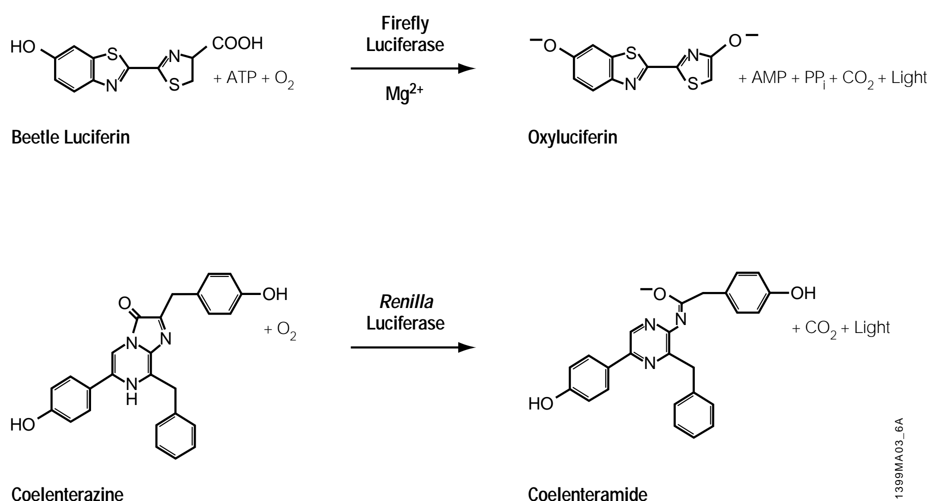

Figure 1. Bioluminescent reactions catalyzed by firefly and *Renilla* luciferases.

Many of Promega's Luciferase Assay Reagents[a,c] 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 stabilized 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 O_2 and coelenterate-luciferin (coelenterazine) (Figure 1). In the DLR™ Assay chemistry, the kinetics of the *Renilla* luciferase reaction provide a stabilized 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 six 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 versus single-sample) used.

An inherent property of coelenterazine is that it emits low-level autoluminescence in aqueous solutions. Originally this drawback prevented sensitive determinations at the lower end of enzyme concentration. Additionally, 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 proprietary chemistry that reduces autoluminescence to a level that is not measurable for all but the most sensitive luminometers. Passive Lysis Buffer 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.

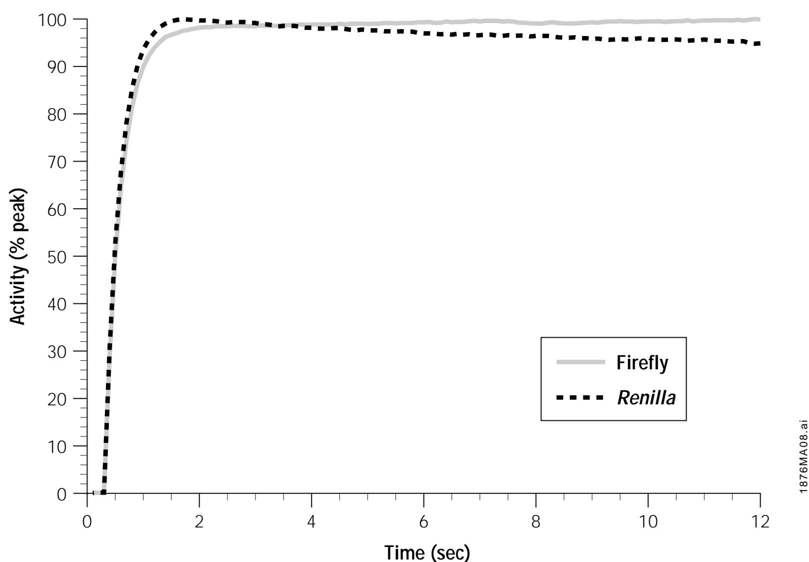

Figure 2. Luminescent signals generated in the Dual-Luciferase® Reporter Assay System[a,b,c] by firefly and *Renilla* luciferases.

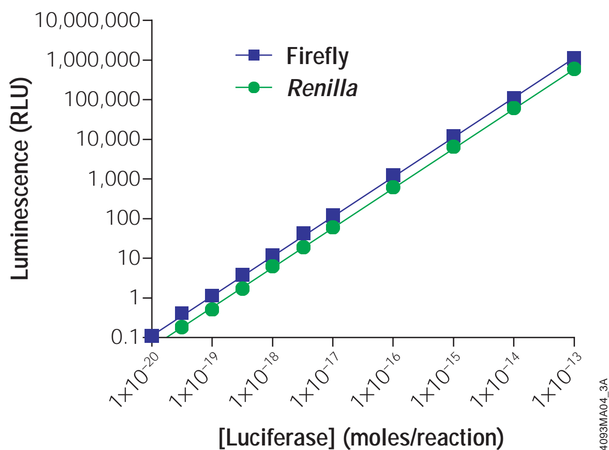

Figure 3. Comparison of the linear ranges of firefly and *Renilla* luciferases. The DLR™ Assay was performed with a mixture of purified firefly and *Renilla* luciferases prepared in PLB containing 1mg/ml gelatin. A Turner Designs Model 20e Luminometer was used to measure luminescence. As shown in this 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 firefly luciferase reporter enzyme. The *Renilla* luciferase assay has a linear range covering six orders of magnitude and allows for the detection of approximately 30 femtograms (approximately 3×10^{-19} moles) of *Renilla* luciferase.

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 10^5 (to ≤0.001% 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:

		Elapsed Time
Step 1:	Manually add prepared lysate to Luciferase Assay Reagent II predispensed into luminometer tubes; mix.	~3 seconds
Step 2:	Quantify firefly luciferase activity.	12 seconds
Step 3:	Add Stop & Glo® Reagent; mix.	3 seconds
Step 4:	Quantitate *Renilla* luciferase activity.	12 seconds
Total elapsed time for the DLR™ Assay		**30 seconds**

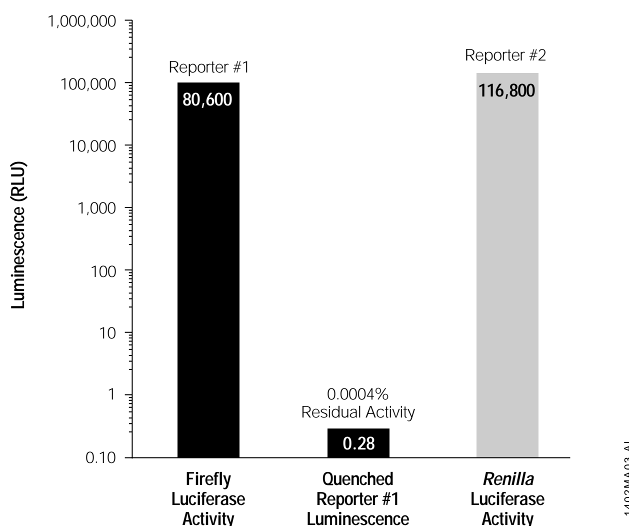

Figure 4. Measurement of luciferase activities before and after the addition of Stop & Glo® Reagent. The DLR™ Assay allows sequential measurement of firefly luciferase (Reporter #1), followed by *Renilla* luciferase activity (Reporter #2) on addition of Stop & Glo® Reagent to the reaction. Both reporter 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[g] (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.

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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 multiwell 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. Other lysis buffers (e.g., Glo Lysis Buffer, Cell Culture Lysis Reagent and Reporter Lysis Buffer) either increase background luminescence substantially or are inadequate for passive lysis. 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.

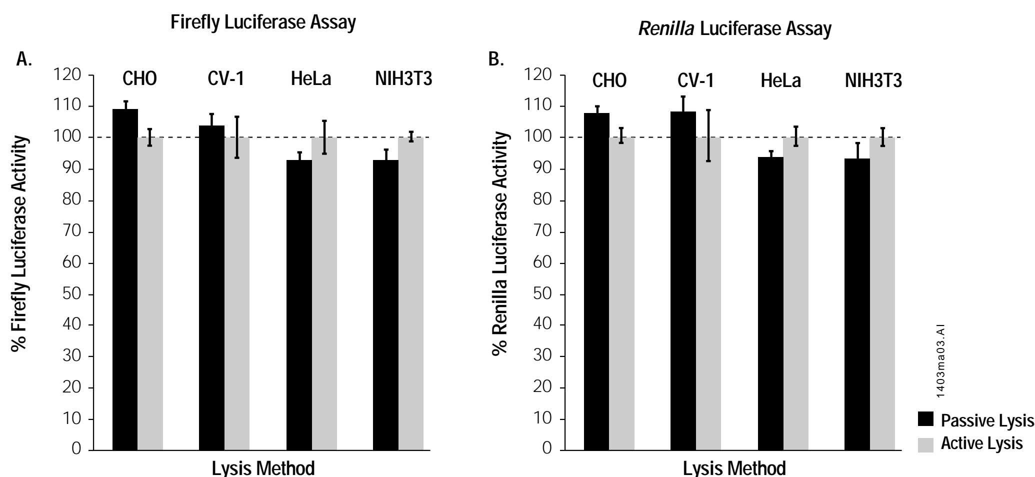

Figure 5. Comparison of firefly luciferase and *Renilla* luciferase reporter activities in cell lysates prepared with Passive Lysis Buffer using either the passive or active lysis procedure. Four different mammalian cell types were co-transfected with a firefly luciferase expression vector and a *Renilla* 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)
- 10ml Stop & Glo® Buffer
- 200µl Stop & Glo® Substrate, 50X
- 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 × 10ml Stop & Glo® Buffer
- 10 × 200µl Stop & Glo® Substrate, 50X
- 30ml Passive Lysis Buffer, 5X
- 1 Protocol

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

Storage Conditions: Upon receipt of the Dual-Luciferase® Reporter Assay System, store at it –20°C. Once the Luciferase Assay Substrate has been reconstituted, it should be divided into working aliquots and stored at –20°C for up to 1 month or at –70°C for up to 1 year. Ideally, Stop & Glo® Reagent (Substrate + Buffer) should be prepared just before each use. If necessary, this reagent may be stored at –20°C for 15 days with no decrease in activity. If stored at 22°C for 48 hours, the reagent's activity decreases by 8%, and if stored at 4°C for 15 days, the reagent's activity decreases by 13%. The Stop & Glo® Reagent can be thawed at room temperature up to 6 times with ≤15% decrease in activity.

III. The phRL and pRL Families of *Renilla* Luciferase Reporter Vectors

A. Description of phRL and pRL Vectors

The phRL and pRL families of *Renilla* luciferase reporter vectors contain cDNA encoding *Renilla* luciferase (R*luc*)(g) cloned from the anthozoan coelenterate *Renilla reniformis* (5). Both series of *Renilla* luciferase reporter vectors code for essentially the same protein and can be used either as the experimental or control reporter gene.

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

The DNA coding for the *Renilla* luciferase within the pRL Vectors is the native DNA sequence containing minor modifications for convenience as a genetic reporter. The DNA coding for the *Renilla* luciferase within the phRL Vectors, however, is a synthetic sequence that has been codon-optimized for use in mammalian cells and has had many transcriptional signaling sequences removed (for details please see Technical Manual TM237 for the phRL Vectors or Technical Bulletins TB237, TB238, TB239 and TB240 for the pRL Vectors). The proteins coded for by the phRL and pRL genes differ by only one amino acid, which is located near the N-terminus of the protein.

B. Important Considerations for Co-Transfection Experiments

Firefly and *Renilla* luciferase vectors may be used together to co-transfect mammalian cells. Either firefly or *Renilla* luciferase may be used as the control or the experimental reporter gene, depending on the experiment and the genetic contructs available. However, it is important to realize that *trans* effects between promoters on co-transfected plasmids can potentially affect reporter gene expression (6). 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.

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 control reporter vector to provide low-level, constitutive expression of that 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.

IV. Instrument Considerations

A. Single-Sample Luminometers

Renilla and firefly luciferases both exhibit stabilized 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.

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 or preparing the lysates directly in each well. Dual-reporter assays are then performed according to the following steps: i) inject Luciferase Assay Reagent II; ii) measure firefly luciferase activity; iii) inject Stop & Glo® Reagent and; iv) 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 may be able to perform DLR™ Assays using elapsed premeasurement and measurement times that are significantly shorter than those prescribed in the standard assay protocol.

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

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 the luminometer does not provide a warning, 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[e], 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 on determining assay background measurements.

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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 multiwell 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 of the prepared lysates at –20°C is suitable for short-term storage (up to 1 month); 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)

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

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, we recommend preparing the volume of PLB required just before use. The 5X PLB should be stored at –20°C.

B. Passive Lysis of Cells Cultured in Multiwell Plates

1. 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.
2. Dispense into each culture well the minimum volume of 1X PLB that is required to completely cover the cell monolayer. The recommended volumes of PLB to be added per well are as follows:

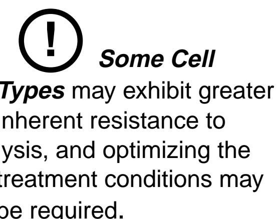
Multiwell Plate	1X PLB
6-well culture plate	500µl
12-well culture plate	250µl
24-well culture plate	100µl
48-well culture plate	65µl
96-well culture plate	20µl

3. 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.
4. 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 made, we recommend clearing the lysate samples for 30 seconds by centrifugation at top speed in a refrigerated microcentrifuge. Transfer cleared lysates to a new 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 (7); 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 (Figure 5). However, some types of cultured cells may exhibit greater inherent resistance to lysis, and optimizing the treatment conditions may be required.



Some Cell Types 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. Homogeneous lysates may be rapidly prepared by manually scraping the cells from culture dishes in the presence of 1X PLB. Recommended 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 homogeneous 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 made, we recommend clearing the lysate samples for 30 seconds by centrifugation in a refrigerated microcentrifuge. Transfer the cleared lysates to a fresh tube prior to reporter enzyme analyses.

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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

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

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 freeze-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

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

Stop & Glo® Reagent (Substrate + Buffer) is best when prepared just before use. If stored at 22°C for 48 hours the reagent's activity decreases by 8%. If necessary, Stop & Glo® Reagent may be stored at –20°C for 15 days with no decrease in activity. It may be thawed at room temperature up to 6 times with ≤15% decrease in activity.

Example 1 (10 assays):

Add 20µl 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 for 10 assays.

Example 2 (100 assays):

Transfer 10ml Stop & Glo® Buffer into a glass vial or siliconized polypropylene tube. Add 200µl of 50X Stop & Glo® Substrate to the 10ml Stop & Glo® Buffer. This will prepare sufficient Stop & Glo® Reagent for 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 phRL and pRL Vector-transfected cells. For this application, we recommend the *Renilla* Luciferase Assay System(c,f) (Cat.# E2810, E2820). 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 the appropriate number of luminometer tubes 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 connected to 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 connected to a printer or computer, record the *Renilla* luciferase activity measurement.
7. Discard the reaction tube, and proceed to the next DLR™ Assay.

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

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.

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.

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, especially if injectors are used for dispensing more than one type of reagent. 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 and that on completion of a run the wash protocol, below, be followed to ensure clean lines. Proper cleaning must be followed even when an injector is dedicated for dispensing a single reagent.

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 of 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.

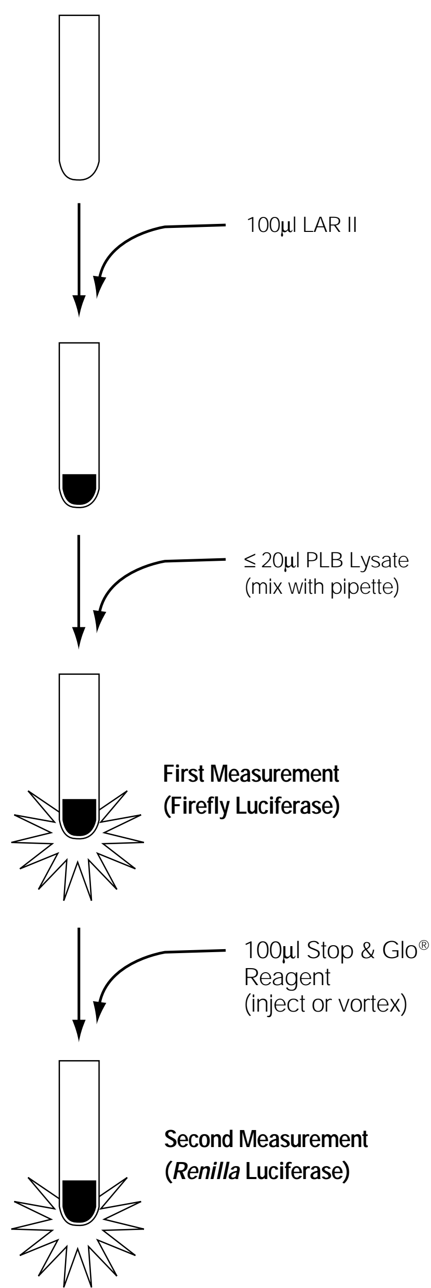

Figure 6. Format of the DLR™ Assay using a manual luminometer or a luminometer equipped with one reagent injector. If the instrument is equipped with two injectors, it may be preferable to predispense the lysate into luminometer tubes, followed by sequential auto-injection of the LAR II and Stop & Glo®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

Firefly Luciferase

With rare exceptions, all background luminescence 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 carefully 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 100µl of LAR II to 20µl of NTC lysate.
3. Measure apparent luminescence activity.

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 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 plate. Be aware that the relative noise in background signals is often quite high. Therefore, 5–10 readings should be performed, and the mean reading used to obtain a statistically significant value for instrument and plate background. An additional source of high luminescence activity is overflow from an adjacent well. This can be eliminated by using high quality opaque plates that prevent cross-talk. Additionally, the luminometer mechanics and its ability to read luminescence emitted from individual wells should be examined before launching an experiment. Each instrument differs in its method of injection and luminescence detection, which can play a significant role in cross-talk.

Renilla Luciferase

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

1. Instrument and sample tube background luminescence, which is similar to that noted above for firefly luciferase.
2. Autoluminescence of coelenterazine 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. Stop & Glo® Reagent has been developed with a proprietary formulation to further reduce autoluminescence. Between the effects of the PLB and the Stop & Glo® Reagent formulations, many luminometers are unable to measure the residual autoluminescence.

3. Residual luminescence from the firefly luciferase reaction 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.

The background luminescence contributed by numbers 1 and 2 above is constant and can be subtracted from all measurements of *Renilla* luciferase. Because the background from number 3 is variable, depending on the expression of firefly luciferase, it may be important to verify that the 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 mix. In addition, if the first injection of LAR II coats the walls of the tube but the second injection with the Stop & Glo® Reagent does not cover the same exposed surface area, inadequate quenching may result.

Perform the following steps to determine the background contributed by the instrument, sample tube and coelenterazine autoluminescence:

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 LAR II.
3. Add 100µl of Stop & Glo® Reagent to the sample tube.
4. Measure background.

Perform the following steps to determine the background from residual firefly luciferase luminescence:

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 LAR 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_2HPO_4
2g KH_2PO_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

Luciferase Assay Systems and Reagents

Product	Size	Cat.#
Dual-Luciferase® Reporter 1000 Assay System[a,b,c]	1,000 assays	E1980
Luciferase Assay System[a,c]	100 assays	E1500
	1,000 assays	E1501
Renilla Luciferase Assay System[c,f]	100 assays	E2810
	1,000 assays	E2820
Dual-Glo Luciferase Assay System[a,b,c,d]	10ml	E2920
	100ml	E2940
	10 × 100ml	E2980
QuantiLum® Recombinant Luciferase[e]	1mg	E1701
	5mg	E1702
Passive Lysis Buffer, 5X	30ml	E1941

Luciferase Reporter Vectors

Product	Size	Cat.#
phRL-null Vector(c,f,g,h)	20µg	E6231
phRL-TK Vector(c,f,g,h)	20µg	E6241
phRL-TK(Int–) Vector(c,f,g,h)	20µg	E6251
phRL-SV40 Vector(c,f,g,h)	20µg	E6261
phRL-CMV Vector(c,f,g,h,i)	20µg	E6271
phRG-B Vector(c,f,g,h)	20µg	E6281
phRG-TK Vector(c,f,g,h)	20µg	E6291
pRL-SV40 Vector(g)	20µg	E2231
pRL-TK Vector(g)	20µg	E2241
pRL-CMV Vector(g,i)	20µg	E2261
pRL-null Vector(g)	20µg	E2271

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

Luminometers

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

[(a)]U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289, European Pat. No. 0 553 234 and Japanese Pat. No. 3171595 have been issued to Promega Corporation for a beetle luciferase assay method, which affords greater light output with improved kinetics as compared to the conventional assay. Other patents are pending.

[(b)]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.

[(c)]Certain applications of this product may require licenses from others.

[(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 under U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

[(f)]Patent Pending.

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

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