TECHNICAL MANUAL

Dual-Luciferase® Reporter Assay System

Instructions for use of Products
E1910 and E1960

Revised 6/15
TM040

Dual-Luciferase® Reporter Assay System

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

1. 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 eliminated. Thus, dual-reporter assays often allow more reliable interpretation of the experimental data by reducing extraneous influences.

The Dual-Luciferase® Reporter (DLR™) Assay System[a–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 simultaneously initiated by 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.

Promega offers the pGL*4* series of firefly and *Renilla* luciferase vectors designed for use with the DLR™ Assay Systems. These vectors may be used to co-transfect mammalian cells with experimental and control reporter genes.

1.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.

Many of our Luciferase Assay Reagents 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).

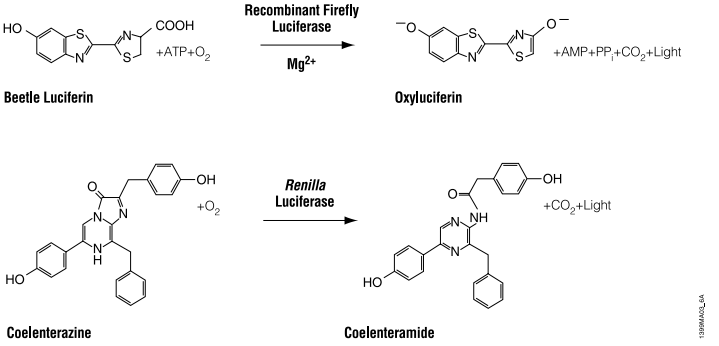

Figure 1. Bioluminescent reactions catalyzed by firefly and *Renilla* luciferases.

1.A. Dual-Luciferase® Reporter Assay Chemistry (continued)

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. The 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.

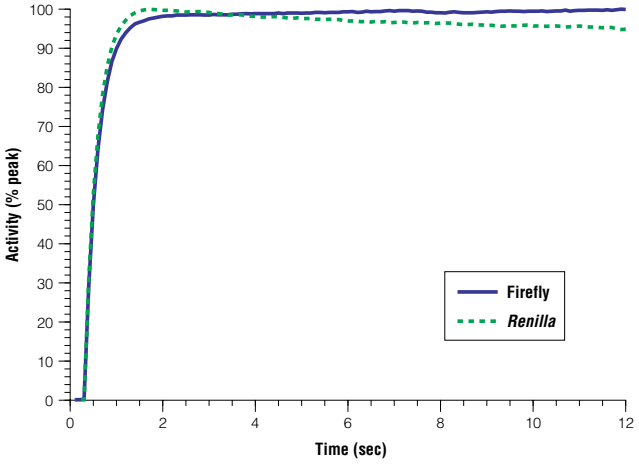

Figure 2. Luminescent signals generated in the Dual-Luciferase® Reporter Assay System by firefly and *Renilla* luciferases.

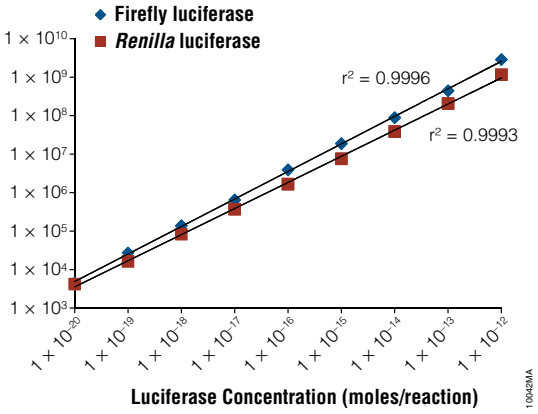

Figure 3. Comparison of the linear ranges of firefly and *Renilla* luciferases. The DLR™ Assay was performed with a mixture of purified firefly and *Renilla* luciferases prepared in PLB containing 1mg/ml BSA. A Promega GloMax® 20/20 Luminometer was used to measure luminescence. As shown in this graph with the DLR™ Assay System, the linear range of the firefly luciferase assay is eight orders of magnitude, providing detection sensitivity of ≤0.1 femtogram (approximately 10^{-21} mole) of firefly luciferase reporter enzyme. The *Renilla* luciferase assay has a linear range covering eight orders of magnitude and allows for the detection of approximately 0.1 femtogram (approximately 10^{-21} mole) of *Renilla* luciferase.

1.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**

1.B. Format of the Dual-Luciferase® Reporter Assay (continued)

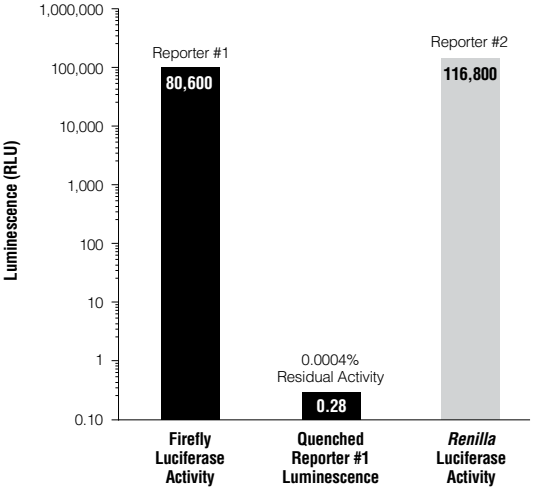

Figure 4. Measurement of luciferase activities before and after the addition of Stop & Glo® Reagent. The DLR™ Assay allows sequential measurement of firefly luciferase (Reporter #1), followed by Renilla luciferase activity (Reporter #2) on addition of Stop & Glo® Reagent to the reaction. Both reporter activities were quantitated within the same sample of lysate prepared from CHO cells co-transfected with pGL3 Control Vector (Cat.# E1741) and pRL-SV40 Vector (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.

1.C. Passive Lysis Buffer

Passive Lysis Buffer (PLB) is specifically formulated to promote rapid lysis of cultured mammalian cells without the need to scrape adherent cells or perform 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.

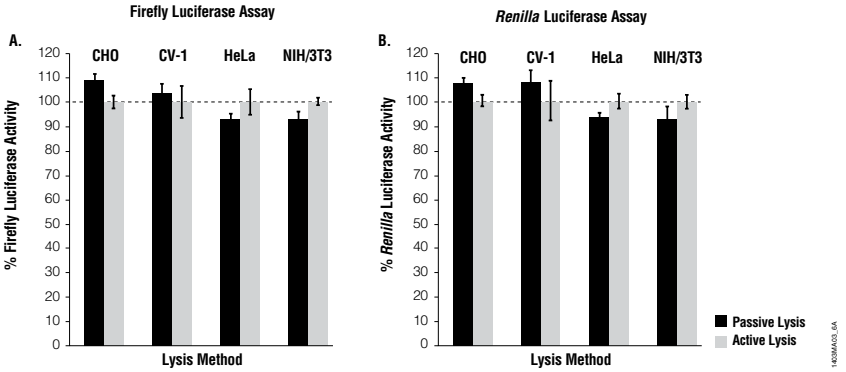

Figure 5. Comparison of firefly and *Renilla* luciferase reporter activities in cell lysates prepared with Passive Lysis Buffer using either the passive or active lysis procedure. Four different mammalian cell types were co-transfected with firefly and *Renilla* luciferase expression vectors. Lysates 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.

2. Product Components and Storage Conditions

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

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

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, store the Dual-Luciferase® Reporter Assay System at –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.

3. The pGL4 Luciferase Reporter Vectors

3.A. Description of pGL4 Vectors

The pGL4 Luciferase Reporter Vectors are the next generation of reporter gene vectors optimized for expression in mammalian cells. Numerous configurations of pGL4 Vectors are available, including those with the synthetic firefly *luc2* (*Photinus pyralis*) and *Renilla hRluc* (*Renilla reniformis*; 5) luciferase genes, which have been codon optimized for more efficient expression in mammalian cells. Furthermore, both the reporter genes and the vector backbone, including the ampicillin (Amp^r) gene and mammalian selectable marker genes for hygromycin (Hyg^r), neomycin (Neo^r) and puromycin (Puro^r), have been engineered to reduce the number of consensus transcription factor binding sites, reducing background and the risk of anomalous transcription.

The pGL4 Vector backbone is provided with either the *luc2* or *hRluc* genes and, in certain vectors, one or both of two Rapid Response™ reporter genes. The protein levels maintained by these Rapid Response™ luciferase genes respond more quickly and with greater magnitude to changes in transcriptional activity than their more stable counterparts.

For more information on advantages of and improvements made to the pGL4 series of vectors, please visit: **www.promega.com/pgl4/** or see the *pGL4 Luciferase Reporters Technical Manual* #TM259.

3.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.

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4. Instrument Considerations

4.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 reagents.

The GloMax® 20/20 Luminometers, equipped with single or dual auto-injector systems (Cat.# E5321 or E5331) are ideally suited for low- to medium-throughput processing of DLR™ Assays. The GloMax® 20/20 Luminometer is preprogrammed 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 individual and normalized luciferase values, as well as statistical analyses of values measured within replicate groups.

4.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, such as the GloMax® 96 Luminometer (Cat.# E6511, E6521). 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 as follows: i) inject Luciferase Assay Reagent II; ii) measure firefly luciferase activity; iii) inject Stop & Glo® Reagent and; iv) measure *Renilla* luciferase activity. Therefore, we recommend multi-sample and plate-reading luminometers be equipped with 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.

Note: Verify that your luminometer provides a diagnostic warning when the luminescence of a given sample exceeds the linear range of the photomultiplier tube. It is common for the luminescence intensity of luciferase-mediated reactions to exceed the linear range of a luminometer. 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., Quanti-Lum® 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 5.A) containing 1mg/ml gelatin. The addition of exogenous protein is necessary to ensure stability of the luciferase enzyme at extremely dilute concentrations.

4.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 6.E for a discussion on determining assay background measurements.

5. 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 8.A.)

- phosphate buffered saline (PBS)

5.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.

Use only Passive Lysis Buffer for the preparation of cell lysates for use with the DLR™ System. PLB is specially formulated to minimize background autoluminescence.

5.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 required to completely cover the cell monolayer. The recommended volumes of PLB to add 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.

5.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

5.C. Active Lysis of Cells by Scraping (continued)

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.

6. Dual-Luciferase® Reporter Assay Protocol

Materials to Be Supplied by the User

- luminometer
- siliconized polypropylene tube or small glass vial

6.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.

Do not substitute Luciferase Assay Reagent (Cat.# E1483), included in the Luciferase Assay Systems (Cat.# E1500, E1501, E4030, E4530, E4550), for LAR II. The Luciferase Assay Reagent supplied with these systems is not designed for use with the DLR™ Assay System.

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., a 1ml aliquot will 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.

6.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 at 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.

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.

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.

6.C. Standard Protocol

Note: The LAR II, Stop & Glo® Reagent and samples should be at ambient temperature prior to performing the Dual-Luciferase® Assay. Prior to beginning this protocol, verify that the LAR II and the Stop & Glo® Reagent have been warmed to room temperature.

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 pGL4 Vector-transfected cells. For this application, we recommend the *Renilla* Luciferase Assay System (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.

6.C. Standard Protocol (continued)

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. **Do not vortex**. Place the tube in the luminometer and initiate reading.

   Note: We do not recommend vortexing the solution at Step 3. 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 6.D) 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.

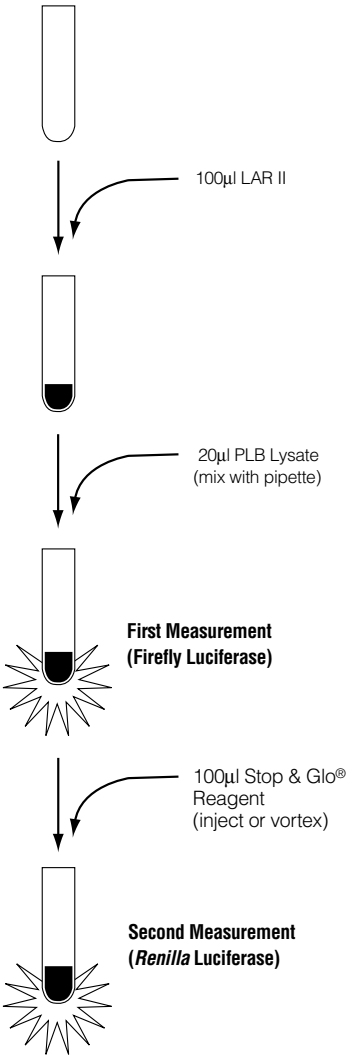

Figure 6. Format of the DLR™ Assay using a manual luminometer or a luminometer equipped with one reagent injector. If the instrument is equipped with two injectors, it may be preferable to predispense the lysate into luminometer tubes, followed by sequential auto-injection of the LAR II and Stop & Glo®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

6.D. Important Considerations for Cleaning Reagent Injectors

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. 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, especially if injectors are used for dispensing more than one type of reagent. 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 performed 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 GloMax® 20/20 Luminometer

Trace contamination of Stop & Glo® Reagent may be removed from the GloMax® 20/20 Luminometer injector system as follows:

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

6.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 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.

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 or tube holding the luciferase reaction. 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 or tube background. An additional source of high luminescence activity is overflow from an adjacent well when measuring luminescence in multiwell plates. 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.

6.E. Determination of Assay Backgrounds (continued)

Renilla Luciferase

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

1. Instrument and sample tube or plate background luminescence 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.

7. References

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8. Appendix

8.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.

8.B. Related Products

Luminometers

Product	Cat.#
GloMax® 20/20 Luminometer	E5311
GloMax® 20/20 Luminometer with Single Auto-Injector	E5321
GloMax® 20/20 Luminometer with Dual Auto-Injector	E5331
GloMax® 96 Microplate Luminometer	E6501
GloMax® 96 Microplate Luminometer with Single Injector	E6511
GloMax® 96 Microplate Luminometer with Dual Injectors	E6521

Luciferase Assay Systems and Reagents

Product	Size	Cat.#
Bright-Glo™ Luciferase Assay System	10ml	E2610
	100ml	E2620
	10 × 100ml	E2650
Steady-Glo® Luciferase Assay System	10ml	E2510
	100ml	E2520
	10 × 100ml	E2550
Dual-Luciferase® Reporter 1000 Assay System	1,000 assays	E1980
Luciferase Assay System	100 assays	E1500
	1,000 assays	E1501
Renilla Luciferase Assay System	100 assays	E2810
	1,000 assays	E2820

Product	Size	Cat.#
Dual-Glo® Luciferase Assay System	10ml	E2920
	100ml	E2940
	10 × 100ml	E2980
EnduRen™ Live Cell Substrate	0.34mg	E6481
	3.4mg	E6482
	34mg	E6485
ViviRen™ Live Cell Substrate	0.37mg	E6491
	3.7mg	E6492
	37mg	E6495
QuantiLum® Recombinant Luciferase	1mg	E1701
	5mg	E1702
Passive Lysis 5X Buffer	30ml	E1941

Plasmid DNA Purification Systems

Product	Size	Cat.#
PureYield™ Plasmid Midiprep System	25 preps	A2492
	100 preps	A2495
PureYield™ Plasmid Maxiprep System	10 preps	A2392
	25 preps	A2393
Wizard® *Plus* SV Minipreps DNA Purification System	50 preps	A1330
	250 preps	A1460

8.B. Related Products (continued)

pGL4 Luciferase Reporter Vectors

Please visit **www.promega.com** to see a complete listing of our reporter vectors.

Vector	Multiple Cloning Region	Reporter Gene	Protein Degradation Sequence	Reporter Gene Promoter	Mammalian Selectable Marker	Cat.#
pGL4.10[*luc2*]	Yes	*luc2*[A]	No	No	No	E6651
pGL4.11[*luc2P*]	Yes	"	hPEST	No	No	E6661
pGL4.12[*luc2CP*]	Yes	"	hCL1-hPEST	No	No	E6671
pGL4.13[*luc2*/SV40]	No	"	No	SV40	No	E6681
pGL4.14[*luc2*/Hygro]	Yes	"	No	No	Hygro	E6691
pGL4.15[*luc2P*/Hygro]	Yes	"	hPEST	No	Hygro	E6701
pGL4.16[*luc2CP*/Hygro]	Yes	"	hCL1-hPEST	No	Hygro	E6711
pGL4.17[*luc2*/Neo]	Yes	"	No	No	Neo	E6721
pGL4.18[*luc2P*/Neo]	Yes	"	hPEST	No	Neo	E6731
pGL4.19[*luc2CP*/Neo]	Yes	"	hCL1-hPEST	No	Neo	E6741
pGL4.20[*luc2*/Puro]	Yes	"	No	No	Puro	E6751
pGL4.21[*luc2P*/Puro]	Yes	"	hPEST	No	Puro	E6761
pGL4.22[*luc2CP*/Puro]	Yes	"	hCL1-hPEST	No	Puro	E6771
pGL4.70[*hRluc*]	Yes	*hRluc*[B]	No	No	No	E6881
pGL4.71[*hRlucP*]	Yes	"	hPEST	No	No	E6891
pGL4.72[*hRlucCP*]	Yes	"	hCL1-hPEST	No	No	E6901
pGL4.73[*hRluc*/SV40]	No	"	No	SV40	No	E6911
pGL4.74[*hRluc*/TK]	No	"	No	HSV-TK	No	E6921
pGL4.75[*hRluc*/CMV]	No	"	No	CMV	No	E6931
pGL4.76[*hRluc*/Hygro]	Yes	"	No	No	Hygro	E6941
pGL4.77[*hRlucP*/Hygro]	Yes	"	hPEST	No	Hygro	E6951
pGL4.78[*hRlucCP*/Hygro]	Yes	"	hCL1-hPEST	No	Hygro	E6961
pGL4.79[*hRluc*/Neo]	Yes	"	No	No	Neo	E6971
pGL4.80[*hRlucP*/Neo]	Yes	"	hPEST	No	Neo	E6981
pGL4.81[*hRlucCP*/Neo]	Yes	"	hCL1-hPEST	No	Neo	E6991
pGL4.82[*hRluc*/Puro]	Yes	"	No	No	Puro	E7501
pGL4.83[*hRlucP*/Puro]	Yes	"	hPEST	No	Puro	E7511
pGL4.84[*hRlucCP*/Puro]	Yes	"	hCL1-hPEST	No	Puro	E7521

[A]*luc2* = synthetic firefly luciferase gene. [B]*hRluc* = synthetic *Renilla* luciferase gene.

9. Summary of Changes

The following change was made to the 6/15 revision of this document:

1. The patent information was updated to remove expired statements.

[a]U.S. Pat. No. 5,744,320 and European Pat. No. 0 833 939.

[b]U.S. Pat. Nos. 7,078,181, 7,108,996 and 7,118,878, European Pat. No. 1297337 and other patents pending.

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