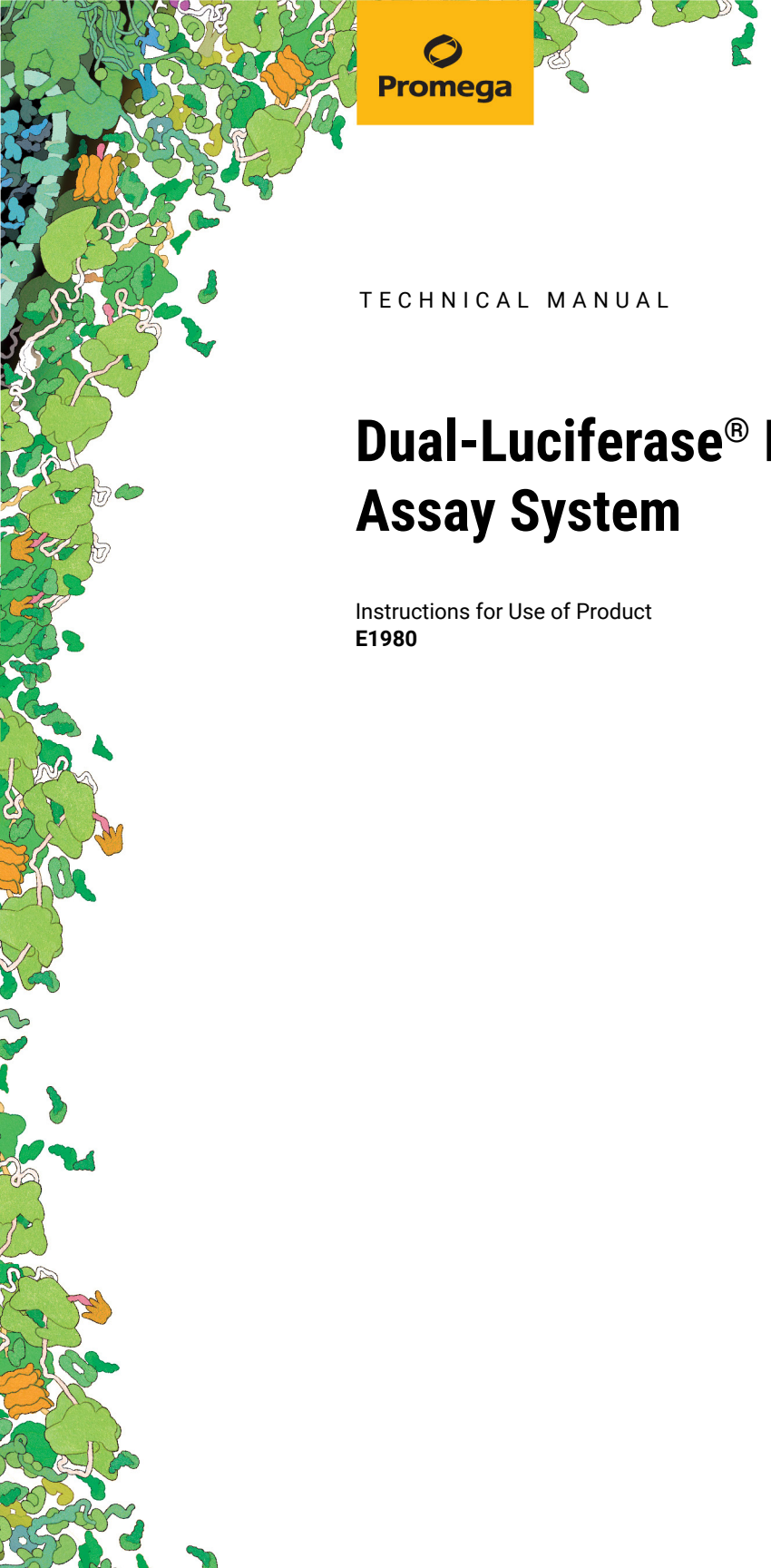

TECHNICAL MANUAL

Dual-Luciferase® Reporter 1000 Assay System

Instructions for Use of Product
E1980

Revised 9/23
TM046

Promega

Dual-Luciferase® Reporter 1000 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

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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 that 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 experimental data by reducing extraneous influences.

The Dual-Luciferase® Reporter (DLR™) Assay System[a,b] provides an efficient means of performing dual-reporter assays. In the DLR™ Reporter 1000 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™ 1000 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™ 1000 Assay provides rapid quantitation of both reporters either in transfected cells or in cell-free transcription/translation reactions.

The DLR™ 1000 Assay System was developed for larger volume users of the DLR™ Assay and is configured for use in 96-well luminometry plates. Additional volume of both assay reagents is supplied to allow priming of reagent injectors. Sufficient lysis reagent (Passive Lysis Buffer, PLB) to allow addition of 20µl/well in 96-well plates is supplied. For applications requiring more lysis reagent (e.g., >100µl/well), additional PLB may be purchased separately (Cat.# E1941). The components of the DLR™ 1000 Assay System are identical in formulation to those provided with the DLR™ Assay System (Cat.# E1910 and E1960).

Promega offers the pGL4 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 bio-luminescent reactions. Thus, using the DLR™ Assay Systems, 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).

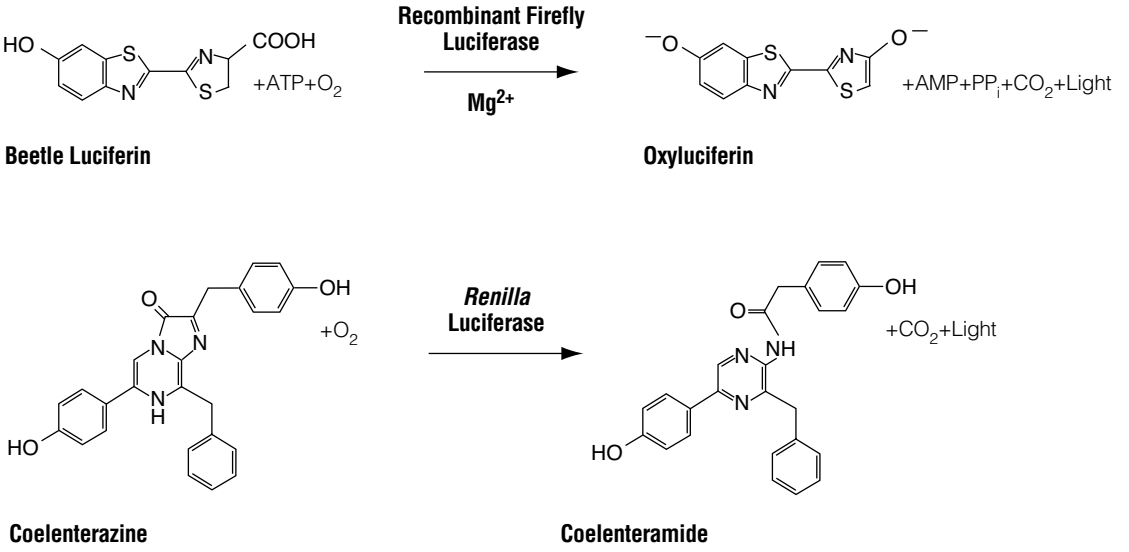

Figure 1. Bioluminescent reactions catalyzed by firefly and *Renilla* luciferases.

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 over 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 vs. 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. Thus, the DLR™ 1000 Assay System allows accurate, reproducible quantitation of very low levels of *Renilla* luciferase activities expressed in prepared cell lysates.

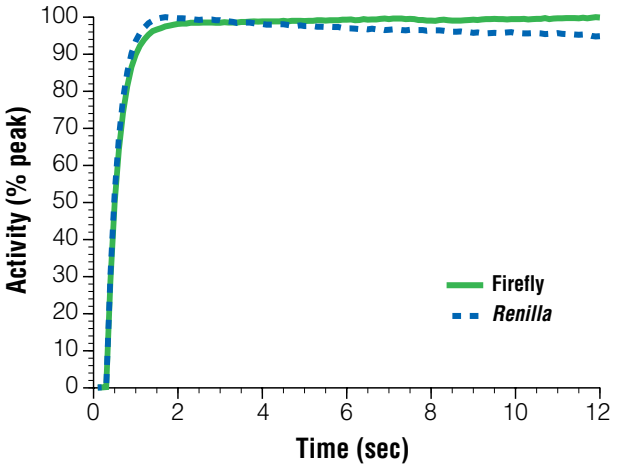

Figure 2. Luminescent signals generated in the Dual-Luciferase® Reporter 1000 Assay System by firefly and *Renilla* luciferases.

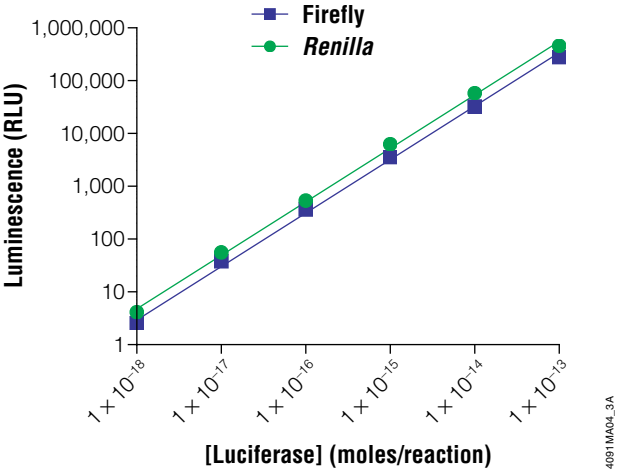

Figure 3. Comparison of the linear ranges of firefly and *Renilla* luciferases. The DLR™ 1000 Assay was performed with a mixture of purified firefly and *Renilla* luciferases prepared in PLB containing 1mg/ml gelatin. A Berthold Orion luminometer was used to measure luminescence. The linear range of each of the luciferase assays using the DLR™ Assay chemistry exceeds six orders of magnitude, providing detection sensitivity ≥30 femtograms (approximately 3×10^{-19} moles) of luciferase using a single-tube luminometer (see *Dual-Luciferase® Reporter Assay System Technical Manual* #TM040). Because plate luminometers are often less sensitive than single-tube luminometers, the minimum measurable luciferase level is often higher than for single-tube luminometers. The luminometer used in this experiment was configured to accommodate measurement of 384-well plates and so showed even lower sensitivity. The photomultiplier tube in the Orion is more sensitive to blue light than to green light, thus the *Renilla* luminescence measurements in this figure appear to be brighter than the firefly luminescence measurements.

1.B. Format of the Dual-Luciferase® Reporter 1000 Assay in 96-Well Plates

The DLR™ 1000 Assay may be performed directly in 96-well plates immediately following lysate preparation without the need to divide samples or perform additional treatments. The DLR™ 1000 Assay also can be performed in single tubes as described in Section 6.D. The firefly luciferase reporter assay is initiated by adding 100µl of Luciferase Assay Reagent II to an aliquot of cell lysate already present in the well. Quenching of firefly luciferase luminescence and concomitant activation of *Renilla* luciferase are accomplished by adding Stop & Glo® Reagent to the well immediately after quantitation of the firefly luciferase reaction. The luminescent signal from the firefly reaction is quenched by at least 10^4 to 10^5 (0.01% to 0.001% residual light output) within 1 second following the addition of Stop & Glo® Reagent (Figure 4). However, the amount of quenching depends on the type of 96-well luminometer used and is a function of the injectors.

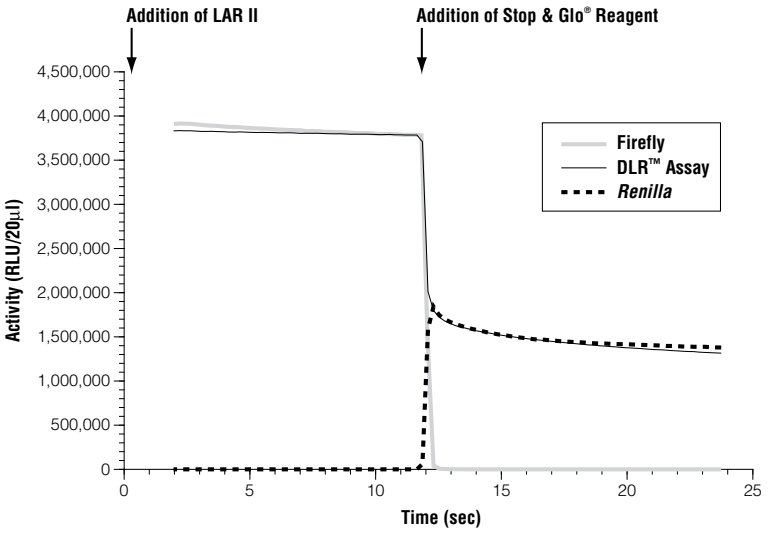

Figure 4. Measurement of luciferase activities before and after the addition of Stop & Glo® Reagent in the presence/absence of luciferin and coelenterazine. The DLR™ 1000 Assay System allows sequential measurement of firefly luciferase activity (Reporter #1) and, immediately following the addition of Stop & Glo® Reagent to the reaction, *Renilla* luciferase activity (Reporter #2). Both enzyme activities are quantitated from 20µl of the same sample containing both purified firefly and *Renilla* luciferases prepared in PLB containing 1mg/ml gelatin. The graph shows the efficient quenching of firefly luciferase by Stop & Glo® Reagent as well as reproducible quantitation of both reporter activities using the DLR™ 1000 Assay System. Firefly luciferase luminescence is quenched without activation of *Renilla* luciferase luminescence by adding Stop & Glo® Buffer that does not contain the substrate for *Renilla* luciferase to the firefly luciferase reaction. Firefly luciferase luminescence is quenched by greater than 5 orders of magnitude. A second signal demonstrates the efficiency of quenching with simultaneous activation of *Renilla* luciferase (DLR™ Assay). The third signal demonstrates that, in the absence of luciferin, firefly luciferase activity is not observed but *Renilla* luciferase is activated using the DLR™ 1000 Assay System.

Complete activation of *Renilla* luciferase is also achieved within this 1-second period. We recommend programming luminometers to provide a 2-second delay followed by a 10-second activity measurement. The assay time may be shortened to a 1- to 2-second delay and a 5-second or less activity read time. The following steps illustrate the format for DLR™ 1000 Assay in 96-well plates using a plate-reading luminometer equipped with two injectors.

Step 1: Addition of 20µl Passive Lysis Buffer to washed cells or addition of prepared lysate to 96-well plates.

Step 2: Injection of 100µl Luciferase Assay Reagent II to quantify firefly luciferase activity.

Step 3: Addition of 100µl Stop & Glo® Reagent, which simultaneously quenches firefly luciferase activity and activates *Renilla* luciferase.

1.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 or performing 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 directly in each well 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. 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 (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 Systems. 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.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Dual-Luciferase® Reporter 1000 Assay System	**1,000 assays**	**E1980**

Each system contains sufficient reagents to prime injectors and perform 1,000 standard Dual-Luciferase® Reporter Assays. The DLR™ 1000 Assay System is developed for use with 96-well luminometry plates. Includes:

- 105ml Luciferase Assay Buffer II
- 1 vial Luciferase Assay Substrate (Lyophilized Product)
- 105ml Stop & Glo® Buffer
- 2 × 1.05ml Stop & Glo® Substrate, 50X
- 1 vial Stop & Glo® Reagent Bottle (empty)
- 30ml Passive Lysis Buffer, 5X

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

Storage Conditions: Store the Dual-Luciferase® Reporter 1000 Assay System between –30°C to –10°C upon receipt. 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)* 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 in combination 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 gene 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.

4. Instrument Considerations

4.A. Plate-Reading Luminometers

The most convenient method of performing large numbers of DLR™ 1000 Assays is to use a plate-reading luminometer capable of processing a 96-well plate such as the GloMax® Navigator (Cat.# GM2000), Discover (Cat.# GM3000) or Explorer System (Cat.# GM3500). For high-throughput applications, we recommend first dispensing 20µl of each sample into the individual wells of the microplate or preparing the lysate directly in each well. The Dual-Luciferase® Reporter 1000 Assay is 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 plate-reading luminometers be equipped with two reagent injectors to perform the DLR™ 1000 Assay. Users of high-throughput instruments may perform DLR™ 1000 Assays using elapsed premeasurement and measurement times that are significantly shorter than those prescribed in the standard assay protocol, such as a 1-second preread delay followed by a 1- to 5-second read time. Note that assay performance can vary substantially depending on the design and operation of the luminometer. We recommend checking the effects of assay delay and read times on luminescence accuracy and for reporter discrimination. The speed and position of the reagent injector can also affect assay performance.

4.A. Plate-Reading Luminometers (continued)

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 your luminometer does not provide such a warning, it is important to establish the linear range of detection of the luminometer prior to performing luciferase reporter assays. Purified luciferase (e.g., QuantiLum® Recombinant Luciferase, Cat.# E1701), or luciferase expressed in cell lysates, may be used to determine the working range of a particular luminometer. Perform serial dilutions of the luciferase sample in 1X PLB (refer to Section 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.B. Single-Sample Luminometers

DLR™ 1000 Assays may be performed with single-sample luminometers for low-throughput applications using the following methods. 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 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.

5. Preparation of Cell Lysates Using Passive Lysis Buffer

Use only Passive Lysis Buffer (PLB) for the preparation of cell lysates. PLB is specially formulated to minimize autoluminescence due to the presence of Stop & Glo® Reagent. The firefly and *Renilla* luciferases contained in cell lysates that are prepared with PLB are stable for at least 6 hours at room temperature (22°C) and up to 16 hours on ice. Freezing of 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. The lysis buffer color may vary due to a naturally derived raw material in the formulation. This color variation does not affect product performance. 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 1 month; however, we recommend preparing the volume of PLB required just before use. The 5X PLB should be stored at –20°C.

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 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 cell lysates to a tube or vial for further handling and storage. For automated applications, perform the DLR™ 1000 Assay directly in the culture plate. In general, it is not necessary to clear lysates of residual cell debris before performing the DLR™ 1000 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 plate prior to reporter enzyme analyses.

Notes:

a. Cultures that are overgrown are often 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 (5); therefore, extending the period of passive lysis treatment will not compromise reporter activities.

b. 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.

5.B. Passive Lysis of Cells Cultured in Multiwell Plates (continued)

Despite the appearance of such cell remnants, we typically find complete solubilization of both luciferase reporter enzymes within a 15-minute treatment period. However, some types of cultured cells may exhibit greater inherent resistance to lysis, and optimizing the treatment conditions may be required.

6. Dual-Luciferase® Reporter 1000 Assay Protocol

Materials to Be Supplied by the User

- plate-reading luminometer

6.A. Preparation of Luciferase Assay Reagent II

Prepare Luciferase Assay Reagent II (LAR II) by resuspending the provided lyophilized Luciferase Assay Substrate in 105ml 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. If the total volume (105ml) of LAR II is not used within a day, LAR II is stable for 1 month at –20°C or for 1 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™ 1000 Assay System.

Notes:

a. Repeated freeze-thaw cycles of LAR II may decrease assay performance. We recommend that LAR II be dispensed into aliquots for each experimental use. Store aliquots at –70°C (or –20°C).

b. The components of LAR II are heat-labile. Frozen aliquots of this reagent should be thawed in a water bath at room temperature.

c. 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

To prepare the reagent, combine the contents of 50X Stop & Glo® Substrate (2.1ml) and 105ml of Stop & Glo® Buffer in the amber Stop & Glo® Reagent bottle provided. Rinse the substrate vial with 2ml from the 105ml Stop & Glo® Reagent to assure complete transfer of the substrate solution. Then add this 2ml rinse back to the vial of Stop & Glo® Reagent. Alternatively, if a smaller volume of Stop & Glo® Reagent will be used, add the 50X Stop & Glo® Substrate to the required volume of Stop & Glo® Buffer to achieve a final concentration of 1X substrate. For example, add 0.2ml 50X Stop & Glo® Substrate to 10ml Stop & Glo® Buffer.

Stop & Glo® Reagent (Substrate + Buffer) is best when prepared just before use. If stored at 22°C for 48 hours the activity of the reagent 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 reagents prior to use because thawing generates density and concentration gradients.

6.C. Assay Protocol for 96-Well Plates

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.

1. Prepare the luminometer to read a 96-well plate according to the following protocol: Set injector 1 to dispense 100µl Luciferase Assay Reagent II. For measurements, use a 1- to 2-second delay and a 5- to 10-second read for firefly luciferase activity. Next, set injector 2 to dispense 100µl Stop & Glo® Reagent, followed by a 1- to 2-second delay and 5- to 10-second read time for *Renilla* luciferase activity.
2. Prepare or dispense 20µl of sample into each well of a 96-well plate.
3. Initialize and prime injectors 1 & 2 with LAR II and Stop & Glo® Reagent, respectively, according to the luminometer instruction manual.

   Note: It is possible to prime auto-injector systems with little or no loss of DLR™ 1000 Assay reagents. Before priming injectors with LAR II or Stop & Glo® reagents, we recommend first purging all storage liquid (i.e., deionized water or ethanol wash solution; see Section 6.E) 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.
4. Insert the 96-well plate, and initiate the plate run.

   Note: We recommend setting injector 1 at a slow dispense speed to avoid splashing in the well. Injector 2 may be set at a higher dispense speed so that adequate mixing of the reagent occurs to quench firefly luciferase activity and initiate *Renilla* luciferase activity. We strongly recommend that the ability of the luminometer for adequate mixing and quenching of firefly luciferase activity be examined. This can be done using the DLR™ 1000 Assay in the absence of Stop & Glo® Substrate. The expected result would be an 80,000- to 100,000-fold reduction in the signal following addition of Stop & Glo® Reagent after subtraction of any background signal due to instrumentation or reagent addition. Although the Stop & Glo® Reagent is designed to inhibit firefly luminescence by greater than 100,000-fold, effective quenching is strongly influenced by the operational characteristics of the luminometer. An efficient microplate luminometer can typically achieve 10,000- to 100,000-fold quenching within 2 seconds of reagent addition. Even though some luminometers may not achieve this level, the effective quenching may still be suitable for the intended application. If the Stop & Glo® Substrate is present, the contribution of autoluminescence must be considered when determining the quenching of firefly luminescence.

6.D. Protocol for Use with Manual or Single-Injector Luminometers

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 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.

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 using the *Renilla* Luciferase Assay System (Cat.# E2810, E2820). If the DLR™ 1000 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. **Do not vortex.** 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 that can escape mixing with the subsequently added volume of Stop & Glo® Reagent. This is of particular concern if Stop & Glo® Reagent is delivered into the tube by automatic injection.
4. If the luminometer is not online with a printer or computer, record the firefly luciferase activity measurement.
5. If available, use a reagent injector to dispense 100µl of Stop & Glo® Reagent. If using a manual luminometer, remove the sample tube from the luminometer, add 100µl of Stop & Glo® Reagent and vortex briefly to mix. Replace the sample in the luminometer, and initiate reading.

   Note: It is possible to prime auto-injector systems with little or no loss of DLR™ 1000 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.E) from the injector system. Priming assay reagent through an empty injector system prevents dilution and contamination of the primed reagent. Thus, the volume of primed reagent may be recovered and returned to the reservoir of bulk reagent.
6. If the luminometer is not online with a printer or computer, record the *Renilla* luciferase activity measurement.
7. Discard the reaction tube, and proceed to the next DLR™ 1000 Assay.

6.E. 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 must be followed to ensure clean lines and a well-maintained instrument. Proper cleaning practices 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 off 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.

6.F. Determination of Assay Backgrounds

The expression of a luciferase reporter is quantitated as the luminescence produced above background levels. Because the background is exceptionally low, luciferase activity is directly proportional to total luminescence in most cases. 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 that are due to firefly and *Renilla* luciferases, as well as cross-talk.

Firefly Luciferase

With rare exceptions, all background luminescence observed in measurements of firefly luciferase arises from the instrumentation or the plates. Background in plates may result from static electricity or from phosphorescence and cross-talk. In particular, polystyrene materials are capable of accumulating significant static buildup that may contribute to persistent, elevated levels of background luminescence. Handling and storage of plates 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 plate:

1. Use Passive Lysis Buffer to prepare a lysate of nontransfected control (NTC) cells.
2. Add 100µl of LAR II to a 20µl aliquot 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. 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 beginning 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 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 well completely, but the second injection of 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, plate 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 into individual wells of a 96-well plate. Inject 100µl of LAR II.
3. Inject 100µl of Stop & Glo® Reagent into the plate.
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 wells of a 96-well plate. Inject 100µl of LAR II.
3. Measure firefly luciferase luminescence.
4. Inject 100µl of Stop & Glo® Reagent.
5. Measure apparent luminescence.
6. Subtract background luminescence contributed by coelenterazine autoluminescence plus instrument and plate background (as determined above).

6.F. Determination of Assay Backgrounds (continued)

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

1. Wood, K.V. *et al.* (1984) Synthesis of active firefly luciferase by in vitro translation of RNA obtained from adult lanterns. *Biochem. Biophys. Res. Comm*. **124**, 592–6.
2. de Wet, J.R. *et al*. (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli. Proc. Natl. Acad. Sci. USA* **82**, 7870–3.
3. Wood, K.V. (1991) In: *Bioluminescence and Chemiluminescence: Current Status,* eds. P. Stanley and L. Kricka, John Wiley and Sons, Chichester, 11.
4. Matthews, J.C. *et al.* (1977) Purification and properties of *Renilla reniformis l*uciferase. *Biochemistry* **16**, 85–91.
5. Sherf, B.A. *et al*. (1996) Dual-Luciferase® reporter assay: An advanced co-reporter technology integrating firefly and *Renilla* luciferase assays. *Promega Notes* **57**, 2–9.
6. Lorenz, W.W. *et al.* (1991) Isolation and expression of a cDNA encoding *Renilla reniformis* luciferase. *Proc. Natl. Acad. Sci.* USA **88**, 4438–42.

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

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 Assay System	100 assays	E1910
	10 × 100 assays	E1960
Luciferase Assay System	100 assays	E1500
	1,000 assays	E1501
Renilla Luciferase Assay System	100 assays	E2810
	1,000 assays	E2820
Dual-Glo® Luciferase Assay System	10ml	E2920
	100ml	E2940
	10 × 100ml	E2980
ONE-Glo™ Luciferase Assay System	10ml	E6110
	100ml	E6120
	1L	E6130
ONE-Glo™ EX Luciferase Assay System	10ml	E8110
	100ml	E8120
	10 × 10 ml	E8130
	10 × 100 ml	E8150
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

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

[A]*luc*2 = synthetic firefly luciferase gene. [B]*hRluc* = synthetic *Renilla* luciferase gene

Vector	Multiple Cloning Region	Reporter Gene	Protein Degradation Sequence	Reporter Gene promoter	Mammalian Selectable Marker	Cat. #
pGL4.80[*hRlucP*/Neo]	Yes	*hRluc*[B]	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]*luc*2 = synthetic firefly luciferase gene. [B]*hRluc* = synthetic *Renilla* luciferase gene

Luminometers

Product	Cat.#
GloMax® Navigator System	GM2000
GloMax® Discover System	GM3000
GloMax® Explorer System	GM3500

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

*For Laboratory Use.

9. Summary of Changes

The following changes were made to the 9/23 revision of this document:

1. Revised Section 5.A.
2. Updated patent statements.
3. Changed font and cover image.
4. Made minor text edits.

Promega

[a]U.S. Pat. Nos. 7,078,181, 7,108,996, 7,118,878 and other patents.

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

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All prices and specifications are subject to change without prior notice.

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