Rapid Luciferase Reporter Assay Systems for High Throughput Studies

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The increased demand for rapid reporter assays has resulted in the introduction of the Luciferase Reporter 1000 Assay System[(a)] *and Dual-Luciferase*™ *Reporter 1000 Assay System*[*(a)]. *These products provide a total of 1,000 assays for either single or dual reporting systems as determined by the user's needs. Both are designed to provide rapid genetic reporter analyses for large-scale experiments and are particularly suited for applications in 96 well luminometry plates. Both systems are adaptable to very short measurements, which allow fast total read times, in an efficient assay format. A 96 well plate can be analyzed in less than seven minutes.*

[(a)]*U.S. Pat. Nos. 5,283,179, 5,641,641 and 5,650,289 have been issued to Promega Corporation for a firefly luciferase assay method, which affords greater light output with improved kinetics as compared to the conventional assay.*

[*]*Patent Pending.*

INTRODUCTION

Bioluminescent reporters of genetic transcription have many applications in cell biology due to the speed, simplicity and precision of their assays. In general, applications have focused primarily on functional analyses of genetic elements, such as promoters and enhancers. However, the range of current uses is much broader, including areas such as receptor function, signal transduction and protein-protein interactions (1). Because the assays are simple and widely applicable, bioluminescent reporters have become increasingly popular as analytical methods for large-scale projects such as drug discovery in pharmaceutical research. To meet this growing demand, Promega has configured its luciferase assay products in larger sizes particularly suited for analyses in 96 well plates.

The new luciferase assay products are the Luciferase Reporter 1000 Assay System (Cat.# E4550) and the Dual-Luciferase™ Reporter (DLR™) 1000 Assay System (Cat.# E1980). Both products have the same assay chemistry as their 100 assay counterparts and provide 1,000 reporter measurements. The Luciferase Reporter 1000 Assay System, for rapid analysis of firefly luciferase expression, is designed for maximal sensitivity. It contains coenzyme A, which provides a stable luminescent signal that decays with a half-life of roughly 10 minutes. The Dual-Luciferase™ Reporter 1000 Assay System integrates this optimized firefly luciferase reaction with a similar assay for *Renilla (Renilla reniformis*, sea pansy coelenterate) luciferase to provide an equally rapid co-reporter assay. Integration of the assay chemistries allows both reporters to be quantitated sequentially in a single sample. Promega's pRL family of *Renilla* luciferase control vectors has been designed for use with the DLR™ Assay. The pRL Vectors[(b)], which provide constitutive expression of *Renilla* luciferase, may be used in combination with any experimental firefly luciferase vector to co-transfect mammalian cells.

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

The 100 assay size Luciferase Assay System (Cat.# E1500) is well known and its performance characteristics are inherent in the Dual-Luciferase™ Reporter System. Therefore, this article will focus primarily on the DLR™ System. Although the Luciferase Assay System has been available for 1,000 assays as a 100ml, stand-alone Luciferase Assay Reagent[(a)] (Cat.# E1483), the new Luciferase Reporter 1000 Assay System is approximately 10% more sensitive. The new system is more sensitive because the substrate is not premixed but is provided lyophilized. The stand-alone Luciferase Assay Reagent is provided frozen and requires lengthy thawing at temperatures below 30°C. In the new Luciferase Reporter 1000 Assay System, the reagent is prepared by hydrating the Luciferase Assay Substrate with the supplied buffer. This buffer can be thawed rapidly at elevated temperatures without compromising the final reagent. The Luciferase 1000 Assay System is supplied without a lysis reagent, and the user may select from a choice of available reagents: Luciferase Cell Culture Lysis Reagent (CCLR, Cat.# E1531), Reporter Lysis Buffer (RLB, Cat.# E3971) or Passive Lysis Buffer (PLB, Cat.# E1941). CCLR provides efficient lysis within minutes; RLB is a mild lysis agent and requires a freeze/thaw cycle to achieve complete cell lysis; and PLB will passively lyse cells without a freeze/thaw cycle. The Dual-Luciferase™ Reporter 1000 Assay System is supplied only with PLB since this reagent is required for optimal performance of the DLR™ System. Additional PLB is available separately, if needed.

CO-REPORTER ASSAYS USING TWO LUCIFERASES

Internal normalization with a co-reporter is a common technique used to improve the reliability and precision of transcriptional analyses with reporters. Traditionally, the beta-galactosidase reporter has been used to normalize the chloramphenicol acetyltransferase (CAT) reporter. However, when firefly luciferase became popular as a reporter, normalization with a co-reporter was cumbersome because of the extreme efficiency of the luciferase assay over other reporters. This problem is solved with Promega's Dual-Luciferase™ Reporter Assay System, which uses a second luciferase from *Renilla reniformis* as the co-reporter. For a list of related references, see Promega's web site at www.promega.com/expression/.

The assays for both luciferase enzymes are rapid and sensitive, and the substrates and chemical mechanisms of these enzymes are completely distinct due to their separate evolutionary origins. Thus, it is possible to integrate the chemistries into a single format where both enzymes can be rapidly quantitated from the same sample (2). Furthermore, the two luciferases are ideal as genetic reporters since they are monomers and do not require post-translational processing for enzymatic activity (3,4). This assay format is well suited for analysis in 96 well plates since the two enzymes can be measured within each well, avoiding the need to divide the samples into separate plates to assay for each co-reporter. Performing a DLR™ Assay in 96 well plates requires a plate-reading luminometer equipped with two reagent dispensers[(c)]. Cell lysates are added to each well (or cells grown in the plates are lysed *in situ*), and the plate is placed in the luminometer. The first dispenser adds the firefly luciferase activating reagent, Luciferase Assay Reagent II (LAR II), and luminescence is recorded. The second dispenser then adds the *Renilla* luciferase-activating reagent, Stop & Glo® Reagent, that simultaneously quenches the firefly luminescence and activates the *Renilla* luciferase. The "glow-type" *Renilla* luminescence then is recorded. The complete dual assay process takes only a few seconds per well.

[(c)] *Assay performance may vary substantially depending on the design and operation of the luminometer. Luminometers displaying a DLR*eady™ *sticker meet or exceed Promega's specifications for optimal performance of the DLR™ Assay.*

DUAL-LUCIFERASE™ REPORTER ASSAY PERFORMANCE

Figure 1 demonstrates performance of the DLR™ Assay (red curve) in a 96 well plate format. In this example, the luminescence during the first 12 seconds comes from firefly luciferase following addition of LAR II, and the luminescence during 13-24 seconds is from *Renilla* luciferase following the addition of Stop & Glo® Reagent. The step-like change in luminescence at 12 seconds shows the rapid exchange that occurs between the activities of firefly and *Renilla* luciferases when the Stop & Glo® Reagent is added. The luminescence signals contributed by each of the luciferases can be exhibited separately by excluding their respective substrates. By excluding coelenterazine, the substrate for the *Renilla* luciferase, from the DLR™ chemistry, luminescence is evident only from firefly luciferase (green curve in Figure 1, 0-12 seconds) and not from *Renilla* luciferase (13-24 seconds). Conversely, by excluding beetle luciferin, the substrate for the firefly luciferase, luminescence is due only to *Renilla* luciferase (blue curve in Figure 1), and not firefly luciferase. The complete DLR™ Assay is the sum of these two incomplete chemistries, where the luminescence of each reporter is distinguished by the timing of reagent additions. In general, a one- to two-second delay is added before each activity measurement to exclude the rapid changes in luminescence that occur due to reagent additions. For example, when the Stop & Glo® Reagent is added to the reaction, the firefly luciferase luminescence is quenched, and the *Renilla* luciferase reaction is initiated. At 12 seconds (Figure 1), these rapid changes in luminescence from the firefly and *Renilla* luciferases may affect activity measurements. However, at 13-14 seconds, virtually all luminescence is due to the *Renilla* luciferase, and the signal decays slowly over the course of the measurement.

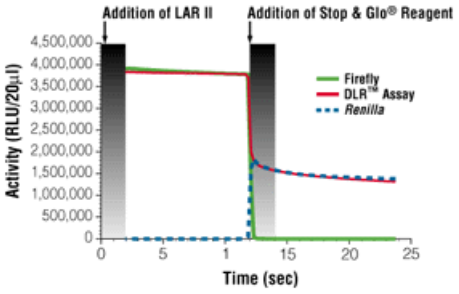

Figure 1. Independence of firefly and *Renilla* luminescence signals. Firefly and *Renilla* luciferases were combined in 20µl Passive Lysis Buffer with 1mg/ml gelatin. Enzymatic activity was measured by the standard Dual-Luciferase™ Reporter Assay (red), Dual-Luciferase™ Reporter Assay without beetle luciferin (blue), and Dual-Luciferase™ Reporter Assay without coelenterazine (green). Luciferase Assay Reagent II (with or without beetle luciferin) was added at time 0, and Stop & Glo® Reagent (with or without coelenterazine) was added at 12 seconds. Each measurement is shown as a 10-second reading with a two-second preread delay period (shaded), thus requiring a total of 24 seconds for both assays. Assays were performed in a 96 well plate using a Berthold® 96V luminometer. RLU = relative light units.

To improve performance in applications using 96 well plates, the DLR™ Assay chemistry was upgraded for both the 100 and 1,000 assay product sizes. The upgrade was designed to make the assay chemistry more compatible for the large surface area typical of automated reagent dispensers, by minimizing surface adsorption of the assay components. Specifically, the quenching agent in Stop & Glo® Buffer was made more hydrophilic, and the concentration of coelenterazine was increased.

This improvement also simplifies the cleaning procedure for the reagent dispenser. After use, the lines are easily cleaned by washing with 70% ethanol. In addition, the upgraded chemistry makes the *Renilla* luminescence approximately two-fold brighter and the signal more stable (Figure 2), similar to the firefly luciferase luminescence.

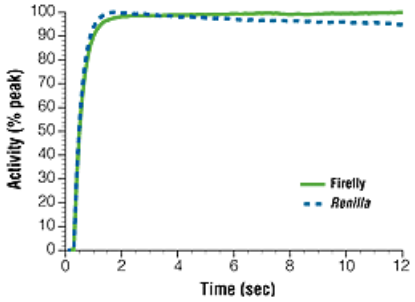

Figure 2. Dual-Luciferase™ Reporter Assay luminescence signals. Firefly and *Renilla* luciferases were combined in 20µl PLB with 1mg/ml gelatin and measured by the standard Dual-Luciferase™ Reporter Assay. The luminescent signals generated were normalized to their respective peak value, which was set at 100. The green signal represents firefly luciferase, and the blue signal represents *Renilla* luciferase.

A titration of the firefly and *Renilla* luciferases shows the sensitivity and linear ranges of the DLR™ Assay (Figure 3). As in the Luciferase Assay System, the assay for firefly luciferase has a sensitivity below 10^{-20} moles and is linear over at least seven orders of magnitude. The assay for *Renilla* luciferase has a sensitivity of 10^{-18} moles and is linear over at least five orders of magnitude. The decreased sensitivity of the *Renilla* luciferase assay is due to autoluminescence caused by nonenzymatic oxidation of coelenterazine. This autoluminescence is enhanced by the presence of protective hydrophobic environments created by some proteins, lipids and detergents. Passive Lysis Buffer (PLB) included with the DLR™ Assay was designed to minimize autoluminescence and to limit it to a constant level in all samples within a set. Thus, the baseline of the *Renilla* luciferase assay is unaffected by endogenous characteristics of the cells themselves. PLB also is designed for applications in 96 well plates by allowing *in situ* lysis of cells without freeze/thaw or scraping. Additionally, PLB contains an agent to minimize foaming during reagent injection by the automated dispensers.

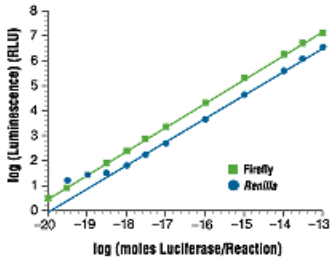

Figure 3. Linear ranges of firefly and *Renilla* luciferases. The Dual-Luciferase™ Reporter 1000 Assay was performed with a mixture of purified firefly and *Renilla* luciferases prepared in Passive Lysis Buffer containing 1mg/ml gelatin. The values shown are averages of three replicate measurements taken over 10 seconds with a two-second preread delay. Assays were performed in a 96 well plate using the Berthold® 96V luminometer. In the measurement of *Renilla* luciferase, the departure from linearity below 10^{-18} moles is due to coelenterazine autoluminescence. No autoluminesence is apparent in measurements of firefly luciferase. RLU = relative light units.

INCREASED PRECISION IN 96 WELL PLATES

Although co-reporters often are used to control for variability in transfection efficiency, they also can be useful for stably transformed cells. This may be particularly true when the cells are grown in very small wells, such as the wells in 96 well plates, where physical parameters, such as "edge effect", can cause variation in gene expression even in clonal cell lines. Figure 4 shows CHO cells assayed 4 hours after seeding into wells of a 96 well plate. The cells were stably transfected to express both the firefly and *Renilla* luciferases from

CMV promoters[d]. From activity of the firefly luciferase alone (Figure 4, Panel A), substantial variation can be seen, suggesting samples with unusually high or low gene expression. However, the apparent expression of the *Renilla* luciferase shows a similar pattern (Figure 4, Panel B). The normalized expression reveals a more uniform pattern (Figure 4, Panel C); thus, normalization to a second reporter can minimize nonspecific variables other than transfection efficiency.

[d]*The CMV vector technology is the subject of U.S. Pat. No. 5,168,062 assigned to the University of Iowa Research Foundation.*

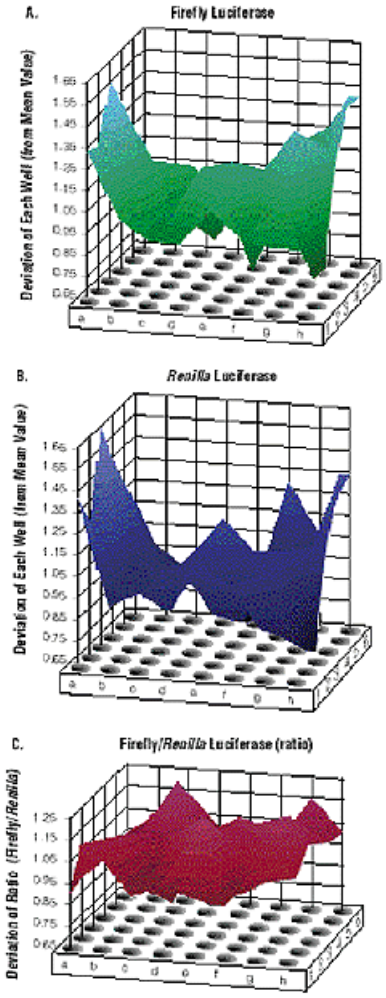

Figure 4. Assay normalization in 96 well plates. The Dual-Luciferase™ Reporter Assay was performed in a 96 well plate on CHO cells stably transfected with both firefly and *Renilla* luciferases. The cells were washed with PBS and then lysed with 20µl of Passive Lysis Buffer per well. Following a 10-minute wait period for lysis to occur, the cells were assayed for both enzyme activities. **Panels A and B** indicate individual luminescence of firefly and *Renilla* luciferases, respectively, per well of the plate. The values given are relative to the mean value, which is arbitrarily set at 1.0. **Panel C** presents the ratio of firefly luciferase activity normalized to *Renilla* luciferase per well. Note the decreased variation of the normalized measurements. Assays were performed in a 96 well plate using a DYNEX MLX™ luminometer.

To attain a relatively high throughput in 96 well plates, it is desirable to minimize the assay time of each sample well. Although a fully manual DLR™ Assay requires about 30 seconds per sample, the time can be greatly reduced with automated reagent dispensers. Table 1, Panel A, shows that the measurement time can be reduced from five to one second for each reporter with little change in the normalized ratio measurement. With the delay times included, the total assay time for each sample can be reduced to four seconds, requiring only 6.5 minutes per plate. The total measurement time can be significantly reduced using a cooled CCD camera, as demonstrated by Wada and colleagues with the Fluorometric Imaging Plate Reader (FLIPR™), which includes an automatic 96 well dispenser (5). Panel B demonstrates that variability in cell density can be normalized with the co-reporter to yield similar ratios within an experimental set. Notice also that the precision of luciferase measurement in every case is improved over two-fold by normalization. Of course, these examples are contrived to indicate only the effects of physical variability in 96 well plates. They do not demonstrate the utility of co-reporters for other variables associated with different experimental treatments, such as the effects of various effector compounds on cellular physiology.

Table 1. Effect of Assay Time on Assay Precision.

A.	**Format 1**	**Format 2**	**Format 3**
	Five Second Read Time*	Three Second Read Time*	One Second Read Tim
Firefly Luciferase Luminescence (RLU)	4,318,640 (±2.5)	2,535,345 (±3.1)	991,926 (±2.9)
Renilla Luciferase Luminescence (RLU)	8,813,396 (±2.4)	5,134,672 (±2.9)	2,166,954 (±3.3)
Ratio of Firefly:*Renilla* Luminescence	0.49 (±0.80)	0.49 (±0.73)	0.46 (±1.1)
B.	**Cells Plated at Low Density***	**Cells Plated at High Density***	
Firefly Luciferase Luminescence (RLU)	90.11 (±17.20)	173.11 (±28.54)	
Renilla Luciferase Luminescence (RLU)	926.71 (±18.10)	1,618.94 (±24.45)	
Ratio of Firefly:*Renilla* Luminescence	0.10 (±9.49)	0.11 (±8.17)	

**Relative standard deviations are shown in parentheses. RLU = relative light units.*

Table 1 demonstrates that the assay time can be considerably reduced without significant loss in experimental accuracy. Panel A shows various time measurements and preread delays using a mixture of the purified luciferases prepared in PLB containing 1mg/ml gelatin. T DLR™ Assay was performed in a 96 well plate using a Berthold® 96V plate reading luminometer (EG&G Wallac, Inc., Gaithersburg, MD), which requires a 1.6-second minimum delay prior to the initial read. The tested time sequences are listed as Formats 1-3. **Format** Two-second preread delay, five-second firefly luciferase measurement; two-second delay, five-second *Renilla* luciferase measurement. **Format 2:** 1.6-second preread delay, three-second firefly luciferase measurement; one-second delay, three-second *Renilla* luciferase measurement. **Format 3:** 1.6-second preread delay, one-second firefly luciferase measurement; 0.5-second delay, one-second *Renilla* luciferase measurement. Panel B represents two different plating densities of stably transfected CHO cells in 96 well plates. The cells w washed with PBS and lysed with 20µl of PLB. After 10 minutes at room temperature, the DLR™ Assay was performed using the DYN MLX™ luminometer (DYNEX Technologies, Inc., Chantilly, VA). The measurements were carried out in 48 replicates with a two-sec delay and five-second read time for each reporter measurement.

SUMMARY

To make luciferase assays more convenient for large-scale users, particularly for applications using 96 well plates, Promega has reconfigured its products into larger sizes. The Luciferase Reporter 1000 Assay System and the Dual-Luciferase™ Reporter 1000 Assay System provide 1,000 assays for bioluminescent reporter and co-reporter measurements. These reporter assays allow rapid and simple quantitation of genetic transcription and other cellular events associated with transcription. The Dual-Luciferase™ Reporter Assay System is particularly useful for minimization of nonspecific assay variability due to physical or biological influences.

ACKNOWLEDGEMENTS

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Ordering Information		
Product	**Size**	**Cat.#**
Luciferase Reporter 1000 Assay System	1,000 assays	E4550

Luciferase Reporter 100 Assay System	100 assays	E1500
Dual-Luciferase™ Reporter 1000 Assay System	1,000 assays	E1980
Dual-Luciferase™ Reporter Assay System, 10-pack	1,000 assays	E1960
Dual-Luciferase™ Reporter Assay System	100 assays	E1910
Luciferase Cell Culture Lysis Reagent, 5X	30ml	E1531
Reporter Lysis Buffer, 5X	30ml	E3971
Passive Lysis Buffer, 5X	30ml	E1941

Related Products		
Product	**Size**	**Cat.#**
pRL-SV40 Vector[b]	20µg	E2231
pRL-TK Vector[b]	20µg	E2241
pRL-CMV Vector[b,d]	20µg	E2261
pRL-null Vector[b]	20µg	E2271

Editor's Note: Promega also offers the pGL3 Luciferase Reporter Vectors, which are designed for functional analysis of genetic regulatory elements. For more information on these vectors, please refer to Product Highlights, page 36.
