

# A New Age of Enlightenment

## New Synthetic *Renilla* Gene and Assay System Increase Expression, Reliability and Sensitivity

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### Abstract

A new synthetic gene encoding *Renilla luciferase* has been designed to provide greater expression efficiency and reliability as a genetic reporter. The synthetic *Renilla* gene (*hRluc*) is codon optimized for mammalian cells and is designed to minimize unintended mechanisms of genetic regulation. The gene is provided in seven different vector configurations to support applications both as a control reporter and a primary reporter. These vectors can be used either with the Dual-Luciferase® Reporter Assay System or the newly developed *Renilla* Luciferase Reporter Assay System. The new *Renilla* Luciferase Reporter Assay System provides high sensitivity due to a novel formulation that virtually eliminates background luminescence common to conventional assays. This new reagent provides rapid and linear quantitation over eight logs of enzyme concentration.

To improve the sensitivity and reliability of the *Renilla* luciferase reporter, the gene sequence was redesigned for optimal performance in mammalian cells.

### Introduction

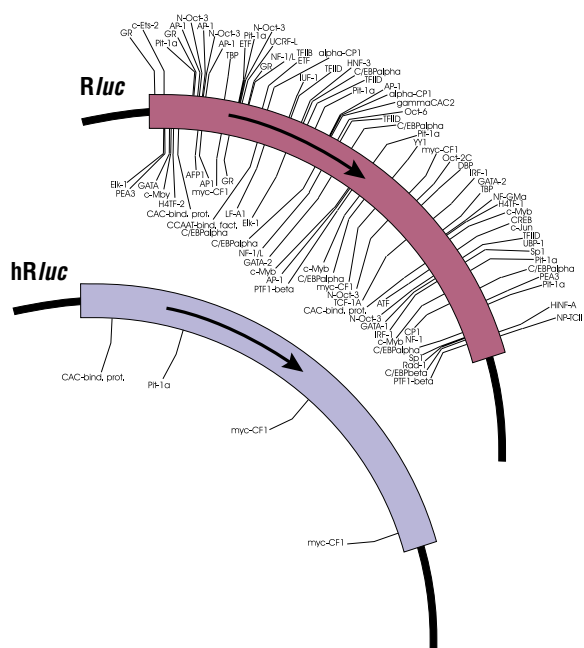
Reporter gene technology has been used as a powerful tool in studying or monitoring gene expression and signal transduction events (1,2). Among several commonly used reporters, the luciferases are frequently preferred because they provide a simple, convenient assay with high sensitivity and a broad range of linearity. Luciferase assays are readily adaptable to different instrumentation and assay formats, making them suitable for routine laboratory measurements and high-throughput screening applications.

*Renilla* luciferase has recently increased in popularity as a genetic reporter, especially in combination with firefly luciferase for dual reporter applications (3). *Renilla* luciferase is a 36kDa monomeric protein that does not require post-translational modification for activity (4). Thus, like firefly luciferase, the enzyme may function as a genetic reporter immediately following translation. In addition, *Renilla* luciferase has distinct potential for in vivo reporting because it does not depend on intracellular magnesium or ATP for the light-generating reaction.

While the enzymological characteristics of *Renilla* luciferase are well suited for reporter applications, the gene in its native form (*Rluc*)<sup>(a)</sup> suffers inherent limitations and potential problems. The gene was cloned from sea pansy (*Renilla*

*reniformis*; 5), so its codon usage is not optimal for expression in mammalian cells. In fact, about 10% of its codons are those least used by mammals. Consequently, the best possible sensitivity as a genetic reporter may not be achieved using the native gene. In addition, transcription factor binding sites in the gene that are appropriate for expression in its native environment could yield unanticipated behaviors in mammalian systems. Analysis of the *Renilla* luciferase gene reveals the presence of many consensus sequences for binding mammalian transcription factors. These consensus sequences could produce anomalous transcriptional behavior in mammalian cells under some conditions, which would compromise the gene's reliability as a reporter molecule.

To improve the sensitivity and reliability of the *Renilla* luciferase reporter, the gene sequence was redesigned for optimal performance in mammalian cells. In particular, preferred mammalian codons were selected for inclusion in the synthetic gene (see Table 1; 6) and mammalian transcription factor binding sites were largely removed



**Figure 1. Illustration of consensus transcription factor binding sites present in the native *Renilla* luciferase and in the synthetic gene.** The synthetic *Renilla* luciferase gene (*hRluc*) and the native gene (*Rluc*) are presented with an approximation of their consensus sequence transcription factor binding sites. About half of the sites found in the native gene are shown in this figure due to space limitations.

(Figure 1). The resulting synthetic *Renilla* luciferase gene (*hRluc*) confers better sensitivity and reliability, extending its capacity as either a primary or control reporter. The nucleotide sequence of the new synthetic gene differs substantially from the native gene, retaining only 72% homology. Nevertheless, the amino acid sequence of the encoded *Renilla* luciferase is virtually unchanged (the second amino acid was changed from Thr to Ala to allow for a consensus Kozak initiation sequence).

The new synthetic *Renilla* luciferase gene is particularly useful when combined with firefly luciferase for dual reporter applications. This combination allows one luciferase to be used as a control for potential sources of experimental variability that may affect accurate analysis of the other reporter. Usually the firefly luciferase serves as the primary reporter and the *Renilla* luciferase as the control reporter. In some cases, both luciferases may be used as primary reporters, each coupled to distinct promoters of experimental consequence.

Promega developed the Dual-Luciferase® Reporter (DLR™) Assay System<sup>(b,c,d)</sup> for efficient quantitation of both firefly and *Renilla* luciferases from a single sample. To perform the DLR™ Assay, a first assay reagent is added to the sample, initiating the firefly bioluminescence reaction. Following quantitation of the luminescence, a second reagent addition quenches the firefly bioluminescence and initiates the *Renilla* bioluminescence. Both assay reactions are sensitive and linear for several logs of enzyme concentration.

Recently Promega introduced a new *Renilla* Luciferase Assay System<sup>(b,e)</sup> for independent measurements of *Renilla* bioluminescence. This new assay reagent provides the highest available sensitivity for quantitation of *Renilla* luciferase. Unlike firefly bioluminescence, assay conditions for *Renilla* luciferase generally yield background luminescence. This luminescence in the absence of enzyme, termed autoluminescence, limits the sensitivity of *Renilla* assays. Although the DLR™ System was formulated to minimize autoluminescence, minimal levels remain. Consequently, in the DLR™ System the sensitivity of firefly luciferase is approximately 10-fold greater than that of *Renilla* luciferase. However, the new *Renilla* Luciferase Assay System is based on a novel formulation that virtually eliminates autoluminescence. The new assay also provides greater luminescent intensity. Combined, these effects yield unsurpassed assay sensitivity. As with other luciferase assays, this new assay is rapid and linear over several logs of enzyme concentration.

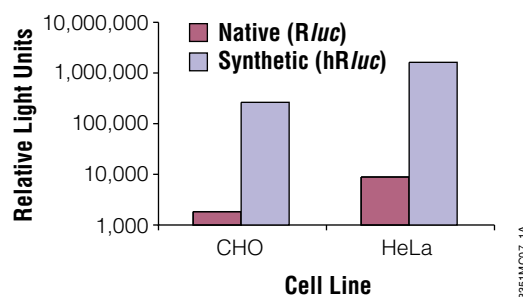
### Synthetic *Renilla* Luciferase as a Control Reporter

There are many circumstances when using reporter genes where a reference "baseline" for expression is required. For example, when performing transient transfections with a reporter gene, a baseline is needed to compensate for vari-

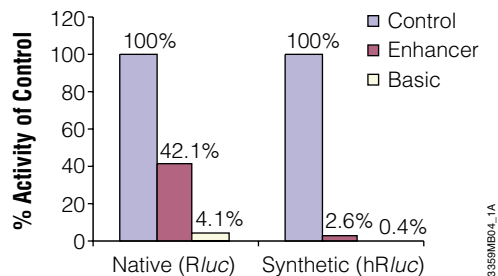
able efficiencies in the transfection process. Or when treating cells with various physiological stimuli, a baseline may be needed to correct for nonspecific effects on cell viability or metabolic activity. Under these circumstances, a second reporter gene is commonly included to provide the baseline measurement from which to compare the expression of the primary reporter. The second reporter gene thus serves as the control reporter. Typically, the control reporter is coupled to a constitutive promoter to provide a constant level of expression that is independent of genetic regulatory mechanisms associated with the primary reporter.

To improve the performance of *Renilla* luciferase as a control reporter, Promega has developed a second generation of vectors containing the synthetic *Renilla* luciferase gene. Two sets of control vectors have been developed, the pHRL<sup>(a,b,e)</sup> and pHRG<sup>(a,b,e)</sup> vector series, each having various configurations to suit differing research needs. Compared to previous control vectors containing the native *Renilla* luciferase gene, significant performance improvements have been achieved, including:

- **Increased reporter expression.** The increased expression efficiency in mammalian cells of the synthetic *Renilla* luciferase gene compared to the native gene results in greater reporter sensitivity (Figure 2). Although the degree of improvement is dependent on the cell type, promoter, and vector backbone, typically expression was increased about 10-fold. In some cases, expression was increased by several hundred-fold. To allow a choice of the most appropriate promoter under differing experimental conditions, the new control vectors are provided with a variety of promoters.
- **Reduced risk of anomalous reporter expression.** Cryptic sites in a gene which may be activated by the experimental conditions would compromise the gene's function as a reporter. The design of the synthetic *Renilla* luciferase gene reduces this risk by minimizing the number of potential



**Figure 2. The synthetic *Renilla* luciferase gene supports higher expression than the native *Renilla* gene in mammalian cells.** CHO and HeLa cells were transfected with pGL3-Control Vector<sup>(g,h)</sup> (with SV40 promoter and enhancer) harboring either the synthetic (*hRluc*) or native (*Rluc*) gene. Cells were harvested 24 hours after transfection and *Renilla* luciferase activity assayed using the Dual-Luciferase® Reporter Assay System.

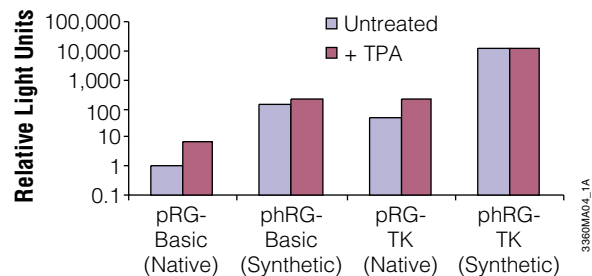


**Figure 3. Reduced anomalous expression from synthetic *Renilla* luciferase gene (*hRluc*).** The synthetic *Renilla* luciferase gene (*hRluc*) and the native gene (*Rluc*) were cloned into a pGL3-Basic Vector<sup>(g,h)</sup> (without promoter or enhancer), a pGL3-Enhancer Vector<sup>(g,h)</sup> (with SV40 enhancer and no promoter) and a pGL3-Control Vector (with SV40 promoter and enhancer) replacing the firefly luciferase gene. These vectors were co-transfected into CHO cells; expression levels are shown as a percentage of corresponding control vectors. The data show that background expression (i.e., measurable luminescence in the absence of a promoter) is greatly reduced by the synthetic reporter. Basal expression from the pGL3-Basic Vector with *hRluc* was reduced by 90% and expression from the pGL3-Enhancer Vector with *hRluc* was reduced by 94%. Similar results have been found in HeLa and NIH3T3 cells (data not shown).

binding sites for mammalian transcription factors. The efficacy of this is supported by comparing the expression of the synthetic gene to the native gene in vectors lacking promoter sequences (Figure 3). In the absence of a promoter, a biologically neutral reporter gene would expectedly show relatively little expression. Such expression is typically referred to as the “background expression”. Results show that the background expression of the synthetic *Renilla* luciferase gene is generally about 90% less that of the native gene. This reduction in background expression is especially apparent in the presence of an enhancer sequence, which is thought to amplify the effects of cryptic sites within the gene sequence.

The utility of the synthetic *Renilla* luciferase gene as a control reporter is also demonstrated by its ability to provide a consistent level of expression under different experimental conditions (Figure 4). One experimental condition in which this is evident is treatment with TPA (phorbol-12-myristate-13-acetate) in MCF-7 cells. The effect of TPA on expression of the control reporter is greatly reduced when the synthetic *Renilla* luciferase gene is used. With appropriate vector selection, the effect can be essentially eliminated. Nevertheless, as expected, induction of the primary reporter by TPA is large and independent of the choice of control reporter.

- **Reduced risk of promoter cross talk.** When different expression vectors are introduced into the same cell, the promoter of one vector may affect the behavior of the other. This interdependence between promoters is sometimes referred to as “cross talk”. Typically, cross talk is increased



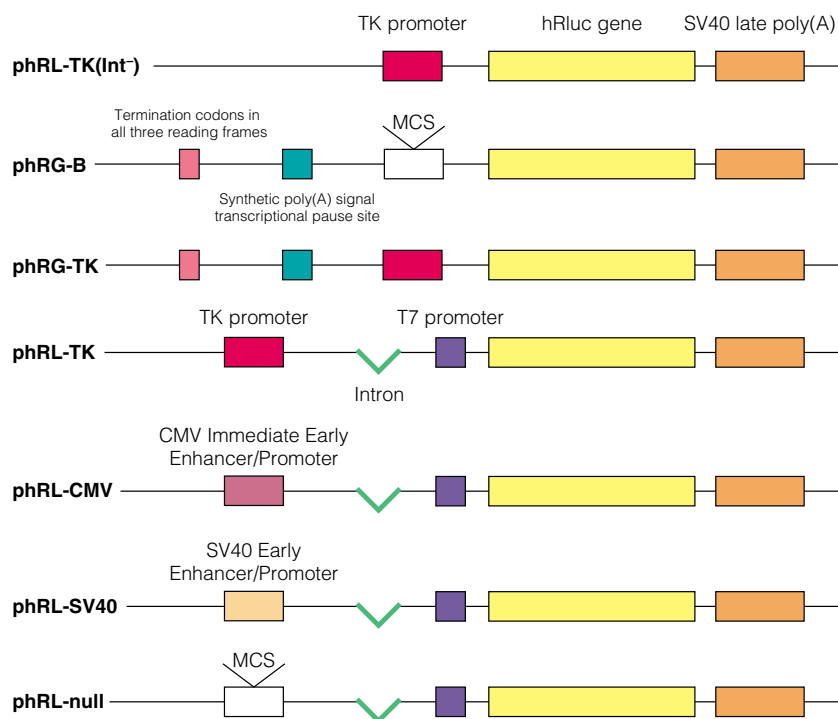
**Figure 4. Synthetic *Renilla* Luciferase Reporter Vectors show improved reliability as control reporters.** pRG-Basic Vectors (without promoter or enhancer) and pRG-TK Vectors (with a minimal thymidine kinase promoter) containing the native gene (*Rluc*) or the synthetic gene (*hRluc*) were transfected into MCF-7 cells. Cells were treated with 0.2nM of TPA (phorbol-12-myristate-13-acetate) or mock-treated with DMSO. Cells were harvested 24 hours after TPA addition and assayed using the Dual-Luciferase<sup>®</sup> Reporter Assay System. Ideally, expression of the control reporter should be immune to non-specific factors. This example shows that control reporter response depends on both the gene and vector sequences. The nonspecific response is reduced with the synthetic *Renilla* luciferase gene in both vector types. Additionally, the nonspecific response is less in the pRG-TK Vectors relative to the pRG-Basic Vectors. In applications using co-reporters, the preferred vector type may depend on experimental conditions.

when large amounts of DNA or strong promoters are used. Because expression from the synthetic *Renilla* luciferase gene has been greatly increased, it may be readily quantitated using less DNA or weaker promoters. When used as a control reporter, this can reduce the risk of cross talk with the primary reporter.

Overall, by increasing expression efficiency and reducing the risk of experimental interferences, the new *Renilla* luciferase vectors confer improved performance as reporter controls. For convenience’s sake, Promega makes these vectors available as transfection-ready DNA. Endotoxin levels have been reduced to <0.1EU/μg DNA, and the DNA quality supports high transfection efficiency into mammalian cells. Because control vectors typically do not require reconfiguration for different experiments, they may be used directly without custom modifications and repurification. This can save time when performing reporter experiments, particularly when making comparisons between the many different vectors available from Promega.

### Choosing A Control Reporter Vector

The seven new reporter vectors containing the synthetic *Renilla* luciferase gene (Figure 5) are configured to provide choices suited to a wide variety of experimental conditions. Two types of vector backbones were used in the design of these vectors: phRL, which supports higher level expression; and phRG, which minimizes background expression originating from the backbone. Specifically, the phRG vec-



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**Figure 5. The new synthetic *Renilla* Luciferase Vectors provide multiple choices in reporter promoter configurations.**

tors contain a poly(A) addition site and translation stop codons in three reading frames located upstream of the promoter or multiple cloning sites. In addition there is no intron in the phRG Vectors, hence expression is lower than that from the phRL Vectors.

To determine the most suitable control vector for a particular application, we recommend testing the vectors under the specific conditions of the experiment. It is important to note whether the control vector adversely affects the expression of the primary reporter and whether the control reporter is inappropriately influenced by experimental conditions. Of course, the general level of expression from the control reporter should also be checked. Generally, expression of the control should be reduced to the lowest level necessary for reliable quantitation.

The phRG-TK Vector<sup>(a,b,e)</sup> is a good choice for a broad range of applications. The TK promoter supports low to medium levels of expression in many cell lines. Also, the reporter gene is contained in the phRG backbone, which is designed to reduce spurious expression. This vector generally provides an appropriate level of reporter expression with good reliability. If expression from the phRG-TK Vector is insufficient, the phRL-TK Vector<sup>(a,b,e)</sup> provides similar characteristics with higher expression. For highest levels of expression, we recommend trying the phRL-SV40<sup>(a,b,e)</sup> or phRL-CMV<sup>(a,b,e,f)</sup> Vectors. Note that with both of these vectors, promoter cross talk (or squelching) can be a problem due to the strong promoter activity. In addition,

they are often influenced by experimental conditions as there are more transcription factor binding sites present in the SV40 and CMV promoters. In particular, the CMV promoter<sup>(f)</sup> is notorious for cross talk with other promoters due to its relatively high number of binding sites.

If the influence of experimental conditions on the expression of the control reporter is a primary concern, the phRL-null<sup>(a,b,e)</sup> and phRG-B<sup>(a,b,e)</sup> Vectors may be suitable alternatives to the phRL-TK and phRG-TK Vectors. The phRL-null Vector has been found to support sufficient expression in some cell lines, and it may be resistant to undesirable interference by some experimental factors (7). The phRG-B Vector expression is usually very low and may not be sufficient without a promoter.

The phRL-TK(Int-) Vector<sup>(a,b,e)</sup> lacks the chimeric intron and can be used in studies involving splicing and RNA transport. To create customized control vectors, the phRL-null and phRG-B Vectors contain multiple cloning sites for inserting other promoters to meet experimental requirements.

### Synthetic *Renilla* Luciferase as a Primary Reporter

The improved *Renilla* luciferase gene will also confer excellent performance for use as a primary reporter. The increase in assay sensitivity and removal of transcription factor binding sites makes the synthetic gene more resistant to influences from experimental conditions not relevant to

regulation of the promoter under study. With these improvements, the synthetic *Renilla* luciferase gene can be used for studying weak promoters or rare events, cells with low transfection efficiency, or to reduce the amount of sample required for assay. The increased reporter expression can also reduce the time required for luminescence measurements, particularly when using CCD-based luminometers. This could greatly increase throughput when assaying large numbers of samples. In some cases, expression of the synthetic *Renilla* luciferase was found to be several-fold greater than firefly luciferase.

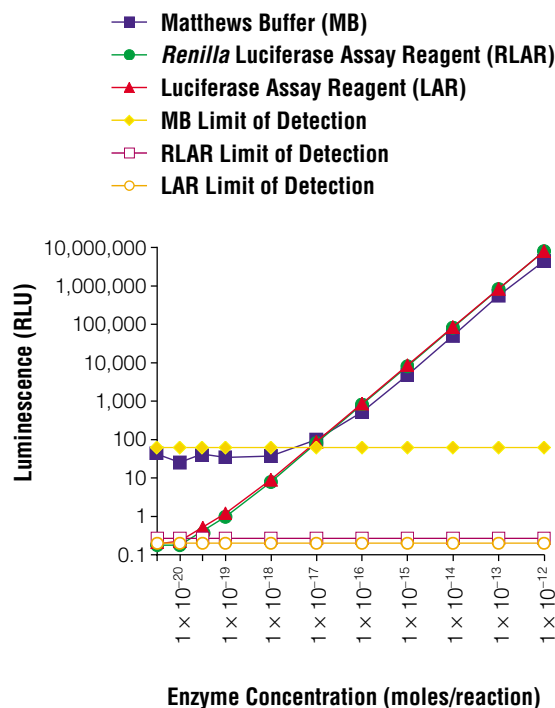
Generally, the pHRG-B Vector should be used for construction of a primary reporter vector. It has the same vector backbone as the pGL3-Basic Vector<sup>(g,h)</sup> containing the firefly luciferase gene. The multiple cloning sites allow insertion of DNA regulatory sequence either upstream or downstream of the *Renilla* luciferase gene. The pHRL-null Vector is not recommended for constructing primary reporter vectors, due to its higher background expression even in the absence of a promoter

## The New *Renilla* Luciferase Assay System

Promega's new *Renilla* Luciferase Assay System is designed to provide a fast, sensitive method for detecting *Renilla* luciferase activity. It may be used to provide an alternative to firefly (*Photinus pyralis*) luciferase as a genetic reporter, or in combination with firefly luciferase for dual reporter applications. The sensitivity of the new assay is greatly improved over conventional assays for *Renilla* luciferase due to a novel formulation that virtually eliminates autoluminescence. The new assay also maximizes the luminescence brightness and is linear over eight logs of enzyme concentration.

Assays for *Renilla* luciferase have generally offered lower sensitivity than comparable assays for firefly luciferase. This is largely because, in the absence of enzyme, assays of firefly luciferase generate no background luminescence, while the autoluminescence associated with *Renilla* luciferase contributes to noise in the assay. The problem is aggravated by hydrophobic molecules, such as lipids and proteins found in cell lysates. Using conventional buffer conditions (e.g., Matthews buffer; 4) in the presence of detergents necessary for cell lysis, the autoluminescence of a *Renilla* luciferase assay could exceed by 100-fold the background in a firefly luciferase assay. Thus, although *Renilla* luciferase serves as an excellent control reporter in combination with firefly luciferase, it was not generally preferred as a primary reporter.

However, by virtually eliminating autoluminescence, the new *Renilla* Luciferase Assay Reagent is analogous in performance to the Luciferase Assay Reagent developed previously for firefly luciferase (Figure 6). In combination with the new synthetic genes for *Renilla* luciferase, this



**Figure 6. *Renilla* Luciferase Assay Reagent has comparable sensitivity to the Luciferase Assay System (firefly) and 100-fold greater sensitivity than a conventional method for assaying *Renilla* luciferase.** The luminescence of *Renilla* and firefly luciferase were compared over a concentration range of  $1 \times 10^{-12}$  to  $3.56 \times 10^{-20}$  moles/reaction. Twenty microliters of *Renilla* luciferase enzyme was diluted in *Renilla* Luciferase Assay Lysis Buffer or Matthews lysis buffer, and 20  $\mu$ l of firefly luciferase enzyme was diluted in Glo Lysis Buffer (Cat.# E2661). The diluted *Renilla* luciferase was then added to 100  $\mu$ l of *Renilla* Luciferase Assay Reagent or Matthews buffer, while the diluted firefly luciferase was added to 100  $\mu$ l of Luciferase Assay Reagent. Light emission was integrated over 10 seconds after an initial 2-second pre-read delay using a Turner Designs Model 20e luminometer. Limits of detection shown represent background plus two standard deviations and were determined for each assay by performing the assay without enzyme. Matthews buffer composition is 0.5M NaCl, 0.1M potassium phosphate, 1.0mM Na<sub>2</sub>EDTA (pH 7.6). Matthews lysis buffer composition is 150mM HEPES (pH 8.0), 0.25% Triton® X-100, 10% glycerol, 289 antifoam, 1mg/ml porcine gelatin.

assay reagent can provide an excellent system for primary reporter applications. In some cases, due to the high expression levels of the synthetic gene, the new *Renilla* Luciferase Assay System could provide significantly greater sensitivity than the firefly luciferase system. Also, by combining this assay with an assay for firefly luciferase, dual reporter applications can be supported. However, such assays would have to be performed independently on separate sample aliquots. This is in contrast to the DLR™ System, where both assays are performed in tandem on a single sample.

## Conclusion

Promega's new vectors containing the synthetic *Renilla* luciferase gene provide greater expression efficiency with reduced risk of anomalous transcriptional behavior. The vectors containing these genes may be used to supply either the control or primary genetic reporters. When used in combinations with firefly luciferase, both luciferases may be efficiently quantitated using the DLR™ System. Alternatively, the *Renilla* luciferase may be quantitated separately using the newly developed *Renilla* Luciferase Assay System. Either assay method provides rapid quantitation with linearity over several logs of reporter concentration. The *Renilla* Luciferase Assay System is particularly sensitive due to improved assay brightness and lower background luminescence.

## References

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2. Naylor, L.H. (1999) *Biochem. Pharmacol.* **58**, 749–757.
3. Sherf, B.A. *et al.* (1996) *Promega Notes* **57**, 2–9.
4. Matthews, J.C. *et al.* (1977) *Biochemistry* **16**, 85–91.
5. Lorenz, W.W. *et al.* (1991) *Proc. Natl. Acad. Sci. USA* **88**, 4438–4442.
6. *Synthetic Renilla Luciferase Reporter Vectors Technical Manual #TM237*, Promega Corporation.
7. Behre, G. *et al.* (1999) *BioTechniques* **26**, 24–26, 28.

## Protocols

- ◆ *Renilla Luciferase Assay System Technical Manual #TM055*, Promega Corporation.  
([www.promega.com/tbs/tm055/tm055.html](http://www.promega.com/tbs/tm055/tm055.html))
- ◆ *Synthetic Renilla Luciferase Reporter Vectors Technical Manual #TM237*, Promega Corporation.  
([www.promega.com/tbs/tm237/tm237.html](http://www.promega.com/tbs/tm237/tm237.html))

## Ordering Information

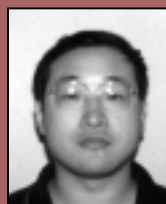
Product	Size	Cat.#
<i>Renilla</i> Luciferase Assay System	100 assays	E2810
	1,000 assays	E2820

## Synthetic *Renilla* Luciferase Reporter Vectors

phRL-null Vector <sup>(i)</sup>	20µg	E6231
phRL-TK Vector <sup>(i)</sup>	20µg	E6241
phRL-TK(Int-) Vector <sup>(i)</sup>	20µg	E6251
phRL-SV40 Vector <sup>(i)</sup>	20µg	E6261
phRL-CMV Vector <sup>(i)</sup>	20µg	E6271
phRG-B Vector <sup>(i)</sup>	20µg	E6281
phRG-TK Vector <sup>(i)</sup>	20µg	E6291

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**Technical Questions?**  
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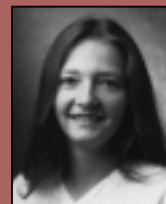
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Keith V. Wood, Ph.D.  
Director, Imaging &  
Reporter Technology.

## As we were going to press...

Promega Notes learned that the Society for Biomolecular Screening has bestowed upon Dr. Keith Wood the "Life Sciences Award for Innovation in Automation and High Throughput Screening". The award, co-sponsored by Perkin Elmer, recognizes Dr. Wood's research and development of bioluminescent reporter gene technology. After joining Promega in 1990, Dr. Wood further developed this technology to enable methods for the rapid and reliable quantitation of >100,000 biological samples per day. This allows researchers to readily evaluate large compound libraries in search of new drugs. According to the SBS, "Dr. Wood's work has led to important discoveries in basic research and has helped propel drug discovery to new frontiers." Dr. Wood will be accepting the award at the annual SBS Conference 2001 in Baltimore, MD, on September 13. More info available online at: [www.sbsonline.org](http://www.sbsonline.org).

<sup>(a)</sup>Licensed under U.S. Pat. Nos. 5,292,658, 5,418,155 and other patents.

<sup>(b)</sup>Certain applications of this product may require licenses from others.

<sup>(c)</sup>U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289, 5,814,471, Australian Pat. No. 649289 and other patents and patents pending.

<sup>(d)</sup>U.S. Pat. No. 5,744,320, Australian Pat. No. 721172 and other patents pending.

<sup>(e)</sup>Patent Pending.

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

<sup>(g)</sup>U.S. Pat. No. 5,670,356.

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

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