

Increased Expression and Convenience with the New pGL3 Luciferase Reporter Vectors

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The pGL3 Vectors are an improved family of reporter vectors utilizing the firefly luciferase gene and based on the previously developed pGL2 Reporter Vectors. The pGL3 Vectors contain the new cytosolic form of luciferase (*luc+*) and multiple changes to the vector backbone to support efficient reporter expression and greater user convenience. These new vectors yield 10- to over 100-fold greater expression of luciferase in mammalian cells, depending on the cell line. Here we describe the rationale underlying the new vector design and present data comparing the pGL3 to the pGL2 Vectors.

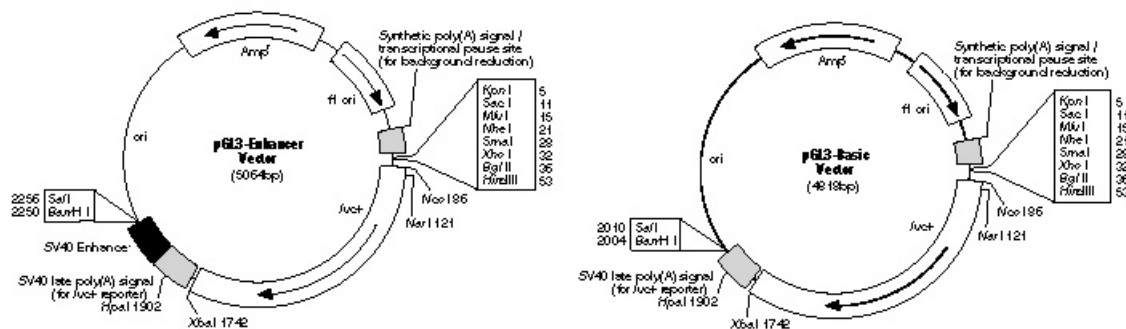
Introduction

The use of reporter genes constitutes a fundamental methodology for the analysis of gene expression and regulation. The types of genetic analyses can be quite varied, including analysis of *cis*-acting mediators (e.g., promoters and enhancers), *trans*-acting mediators (e.g., transcription factors), mRNA processing and translation, and the development of gene delivery methods. To facilitate such experiments, Promega has designed vectors for convenient manipulation and expression of reporter genes in mammalian cells. These vectors include the pCAT® and pGL2 Reporter Vectors, which rely on chloramphenicol acetyl transferase and firefly luciferase, respectively, for genetic reporting.

Here we describe further advancements on the design of the pGL2 Vectors to yield a new family, the pGL3 Luciferase Reporter Vectors. These vectors contain the newly designed cytosolic form of luciferase (*luc+*) and many enhancements to the transcriptional region yielding greater reporter expression. Expression of luminescence has been increased 10- to over 100-fold depending on the cell type. The vectors have also been designed to increase their versatility in different experimental strategies. Combined with Promega's Luciferase Assay Reagent, the pGL3 Vectors provide an optimal system for investigating the molecular biology of gene expression.

Vector structure

Like the pGL2 Vectors, the pGL3 Reporter Vector family consists of four plasmids (see [Figure 1](#)): pGL3-Control, pGL3-Basic, pGL3-Enhancer, and pGL3-Promoter. Each plasmid contains multiple cloning sites upstream and downstream of the reporter gene, a downstream polyadenylation signal for stable mRNA synthesis, and an upstream polyadenylation signal to reduce spurious transcriptional background. All four Reporter Vectors are structurally identical except for the presence or absence of the SV40 enhancer and early promoter elements. The pGL3-Control Vector contains both the enhancer and the promoter which results in strong luciferase expression in many types of mammalian cells. The pGL3-Basic Vector lacks promoter and enhancer sequences, providing maximum versatility for genetic regulatory analysis. The pGL3-Enhancer contains the SV40 enhancer to increase expression from weak promoters; the pGL3-Promoter carries the SV40 promoter for studying putative enhancer elements.



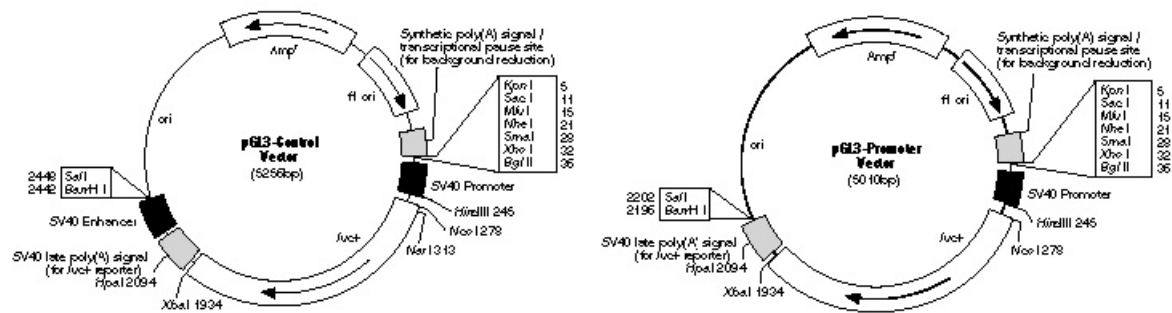


Figure 1. pGL3 Vector Maps.

Description of vector changes

The pGL3 Reporter Vectors differ from their pGL2 predecessors in both reporter gene and vector backbone. These changes were incorporated for three reasons: 1) to increase the efficiency and reliability of reporter gene expression, 2) to reduce non-specific background luciferase expression, and 3) to provide convenient cloning features.

We have optimized reporter gene performance by incorporating elements in the vectors which increase mRNA levels, augment translation efficiency, and improve intracellular localization of luciferase. Spurious transcription originating in the vector backbone has been minimized by incorporating an upstream polyadenylation signal and transcriptional pause site. This modification produces low non-specific background luciferase expression as measured in cells transfected with the pGL3-Basic and Enhancer Vectors. Greater cloning convenience has been provided by incorporating a unique *Nco* I site for luciferase fusion constructions, minimizing vector rearrangements by having two different poly(A) signals in the plasmids, and altering a number of restriction endonuclease sites for easier subcloning. [Table 1](#) provides a complete list of the new pGL3 Vector features.

Table 1. Summary of pGL3 Vector Modifications.

Change from pGL2	Reason

FUNCTIONAL ENHANCEMENTS	

Modifications to the luciferase gene (<i>luc</i> to <i>luc+</i>) (1).	Eliminates peroxisomal targeting sequences, consensus regulatory sequences and inconvenient restriction sites.

Polyadenylation [poly(A)] signal changed from early to late SV40 poly(A) signal.	Yields greater reporter mRNA (4).

Poly(A) site for background reduction changed from SV40 early site to a synthetic poly(A)/transcriptional pause site (5-7).	Avoids possible recombination between homologous SV40 poly(A) sequences present in the same plasmid.

Intron from SV40 small-t removed.	Small-t antigen intron can reduce expression when placed 3' of some genes due to cryptic splicing (13,14).

Kozak consensus sequence created immediately 5' of <i>luc+</i> gene (3).	Provides optimal translation initiation in eukaryotic cells.

Change from pGL2	

CONVENIENCE FEATURES	

<i>Nco</i> I site removed from enhancer and promoter regions and located at 5' end of <i>luc+</i> gene.	Ability to create fusions with the reporter gene using a unique 5' <i>Nco</i> I site.

<i>Sma</i> I site moved to internal position in multiple cloning site (MCS).	Blunt-ended inserts can be ligated into the MCS and restricted on either side by other restriction

endonucleases.

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Xba I site created just          Facilitates insertions into the 3'  
downstream of the luciferase    untranslated region of the mRNA or  
gene.                            subcloning of the luciferase gene.  
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Cytosolic-form luciferase reporter

Several recent modifications to the luciferase gene (*luc+*) optimize its reliability as a general genetic reporter in eukaryotic cells (1). The most important of these is removal of a targeting signal which causes native luciferase translocation into specialized peroxisomes in the firefly photocytic cells. Expression of native luciferase in foreign eukaryotic cells similarly results in peroxisomal translocation. Moreover, higher levels of luciferase expression cause saturation of the peroxisomes with luciferase which drives additional enzyme into the cytoplasm (2). The consequences of this on normal cellular physiology or the expression dynamics of luciferase are unknown. To minimize potential interference, we created a cytosolic form of luciferase, *luc+*, by inactivating the targeting signal. Other modifications to the native luciferase gene increase its general utility as a reporter. These include removal of common internal restriction endonuclease sites and consensus regulatory sites. A complete list of these modifications and a more detailed description of their effects on reporter gene activity are described in reference 1.

Cloning sites

The versatile restriction endonuclease sites of the pGL2 Vectors have been maintained in the pGL3 Vectors with some additional improvements. A multiple cloning site (MCS) region is positioned upstream of the reporter gene containing numerous sites for cloning; the orientation of these sites allows for nested deletion analysis using Exonuclease III (e.g., Promega's Erase-a-Base® System, Cat.# E5850). In the pGL3 Vectors, the *Sma* I site, which is located at the distal end of the MCS in the pGL2 Vectors, is now positioned near the center of the MCS. This feature allows DNA fragments inserted by blunt-ended ligation, which usually destroys the site, to be subsequently subcloned or analyzed using the flanking restriction sites.

In the pGL3 Vectors, a new *Nco* I site spanning the *luc+* initiation codon allows for creation of gene fusions with the reporter and also provides an efficient translation initiation sequence as described by Kozak (3). Removal of two *Nco* I sites, one in the SV40 promoter and one in the SV40 enhancer, make this newly created *Nco* I site unique. In addition to the *Nco* I site, the luciferase gene is flanked by a unique upstream *Hind* III site and a unique downstream *Xba* I site.

Two additional unique cloning sites, *Bam*H I and *Sal* I, located just downstream of the transcribed region, provide convenient cloning sites for *cis*-acting mediators, such as enhancers. The overhanging ends created by *Bam*H I and *Sal* I are compatible with the *Bgl* II and *Xho* I sites upstream of the luciferase gene, providing for positional analysis of the inserted DNA. These downstream sites can also be used for inserting selectable markers (e.g., the *neo* gene) to generate stable transgenic cell lines expressing luciferase.

Polyadenylation signals

The pGL2 and pGL3 Vectors contain two sets of polyadenylation signals, one downstream of the reporter gene and the other upstream of the MCS. The downstream signal directs polyadenylation of the luciferase mRNA for efficient reporter expression. The upstream signal terminates spurious transcription which may originate within the vector backbone.

In the pGL3 Vectors, both polyadenylation signals have been improved to strengthen their functionality. The signals in the pGL2 Vectors are both derived from early SV40 polyadenylation sequences. In the pGL3 Vectors the signal downstream of the luciferase gene has been replaced with the late SV40 signal. This signal has been shown to yield 5-fold greater steady-state levels of mRNA than the early SV40 signal (4). To prevent recombination from occurring between two SV40 signals within the same vector, the upstream signal was replaced by a synthetic poly(A) signal based on the highly efficient poly(A) signal sequence from the rabbit beta-globin gene (5). Following the synthetic poly(A) signal is a transcriptional pause site which has been shown to be associated with termination of transcription (6,7). To further decrease spurious transcription, a short open reading frame with an efficient initiation codon followed by termination codons in all three reading frames has been placed just before the upstream polyadenylation signal. The net effect is increased reporter sensitivity by reducing spurious transcription and translation from the vector backbone.

Intron

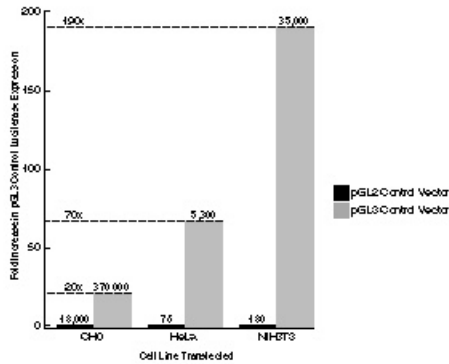
Many eukaryotic expression vectors, including the pGL2 Vectors, contain the SV40 small-t antigen intron with the intent of enhancing gene expression. While flanking intron sequences can often increase the expression of a cDNA insert, this is not always the case (8-12). In particular, the small-t antigen intron placed downstream of a cDNA has been observed to decrease expression due to aberrant splicing (13,14).

We investigated a more efficiently processed chimeric intron containing the 5' splice site of a human beta-globin intron and the branch point and 3' splice site of a human IgG intron (15). The chimeric intron produced a modest increase in luciferase expression (3-fold over no intron) but also caused unexpectedly high background luciferase expression particularly from the pGL3-Enhancer Vector. Therefore, the final design of the pGL3 Vectors does not contain an intron. For researchers interested in using the pGL3 Reporter Vectors for the

study of introns, convenient restriction endonuclease sites flank the luciferase gene.

Performance of pGL2 AND pGL3 reporter Vectors

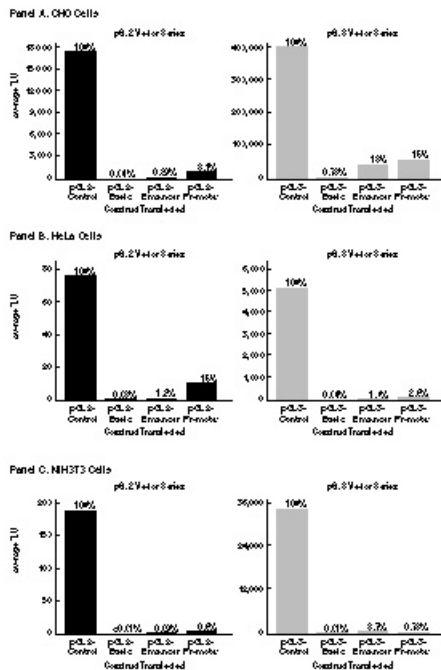
The functionality of the pGL3 Vectors was compared to the pGL2 Vectors by performing transient transfections of HeLa, NIH3T3 and CHO cells. The cells were transfected using calcium phosphate (ProFection® Mammalian Transfection System, Cat. # E1200). Cell lysates were prepared 48 hours later and assayed for luciferase activity (Luciferase Assay System, Cat.# E4030) using a Turner Designs Luminometer. In every case, the new pGL3-Control Vectors produced higher levels of luciferase activity than the pGL2-Control Vectors (Figure 2 shows representative data). Compared to the pGL2-Control, luciferase expression from the pGL3-Control was 50- to 70-fold greater in HeLa cells, 100- to 700-fold greater in NIH3T3 cells, and 20-fold greater in CHO cells.



1.

Figure 2. Comparison of luciferase activities expressed in CHO, HeLa, and NIH3T3 cells transfected with the pGL2-Control and pGL3-Control Reporter Vectors. The expression levels of luciferase are dramatically higher with the pGL3-Control Vector.

Figure 3 shows representative data comparing the performance of the four pGL3 Reporter Vectors to their pGL2 counterparts in CHO, HeLa and NIH3T3 cells. The profiles of the pGL3 and pGL2 Vectors are similar in that they maintain a low level of luciferase activity from the Basic, Enhancer, and Promoter Vectors as compared to the Control Vector. The effective range of expression between the Basic and Control Vectors was comparable for the pGL3 and pGL2 Vectors in HeLa and NIH3T3 cells. In CHO cells, however, the relative difference in luciferase expression from cells transfected with the Control and the Basic vectors was several-fold higher for the pGL2 Vectors than the pGL3 Vectors. A possible reason for this effect in CHO cells is that the already high level of luciferase activity diminishes further expression from the pGL3-Control Vector.



1.

Figure 3. A representative experiment comparing luciferase activities expressed in 3 different cell lines transfected with

the pGL2 and pGL3 Vector series. The increase in luciferase expression observed with the new pGL3 Vectors provides greater sensitivity while maintaining relatively low background luciferase expression.

Figure 3 also shows some subtle differences between the pGL2 and pGL3 Vectors when comparing the luciferase expression levels from the Basic Vectors to their corresponding Enhancer and Promoter Vectors. When considering the substantial differences in the design of the pGL2 and pGL3 Reporter Vectors, subtle changes in the expression patterns observed in different cell types are not surprising. The new pGL3 Vectors contain changes that have altered potential regulatory protein binding sites in both the gene (1) and in the vector. For example, the consensus AP-1 binding site located in the small-t antigen intron of the pGL2 Vectors (16) is absent in the pGL3 Vectors, possibly contributing to relatively increased luciferase expression from the pGL2-Promoter vector in some cells.

Even though attempts have been made to design the pGL3 Reporter Vectors to be transcriptionally neutral, any vector may contain unexpected regulatory sites which could exhibit activities in different cell types. In addition, a given genetic reporter may act differently in various cell types. Hence, it is important that the proper controls be used.

Summary

The pGL3 Reporter Vectors incorporate design changes for the improved performance and convenience of reporter gene expression in mammalian cells. These vectors carry a cytosolic form of firefly luciferase and redesigned functional elements in the vector backbone. The pGL3 Vectors support more efficient expression of luciferase in mammalian cells than the previously developed pGL2 Vectors, making the pGL3 Vectors particularly useful in experiments where greater reporter gene expression is needed.

Acknowledgments

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References

1. Sherf, B.A. and Wood, K.V. (1994) *Promega Notes* **49**, 14.
2. Keller, G.A. *et al.* (1987) *Cell Biol.* **84**, 3264.
3. Kozak, M. (1989) *J. Cell Biol.* **108**, 229.
4. Carswell, S. and Alwine, J.C. (1989) *Mol. Cell. Biol.* **9**, 4248.
5. Levitt, N. *et al.* (1989) *Genes and Devel.* **3**, 1019.
6. Enriquez-Harris, P. *et al.* (1991) *EMBO J.* **10**, 1833.
7. Eggermont, J. and Proudfoot, N.J. (1993) *EMBO J.* **12**, 2539.
8. Callis, J. *et al.* (1987) *Genes and Devel.* **1**, 1183.
9. Gross, M.K. *et al.* (1987) *Mol. Cell. Biol.* **7**, 4576.
10. Buchman, A.R. and Berg, P. (1988) *Mol. Cell. Biol.* **8**, 4395.
11. Evans, M. J. and Scarpulla, R. C. (1988) *Mol. Cell. Biol.* **8**, 35.
12. Huang, M.T.F. and Gorman, C.M. (1990) *Nucl. Acids Res.* **18**, 937.
13. Huang, M.T.F. and Gorman, C.M. (1990) *Mol. Cell. Biol.* **10**, 1805.
14. Evans, M.J. and Scarpulla, R.C. (1989) *Gene* **84**, 135.
15. Brondyk, B. (1994) *Promega Notes* **49**, 7.
16. Kushner, P. J. *et al.* (1994) *Mol. Endo.* **8**, 405.

Ordering Information

Product	Size	Cat. #
pGL3-Basic Vector	20µg	E1751
pGL3-Enhancer Vector	20µg	E1771
pGL3-Promoter Vector	20µg	E1761
pGL3-Control Vector	20µg	E1741
Luciferase Assay System		E1500