



# A New Luminescence: Not Your Average Click Beetle **Promega**

## Introducing Chroma-Luc™ Technology

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### Abstract

The Chroma-Luc™ Reporter Vectors encode luciferases that emit green and red luminescence. Although the luciferases are 98% identical in amino acid sequence, their peak luminescence wavelengths are separated by >75nm. Both colors of luminescence are generated by a single addition of Chroma-Glo™ Reagent, which has been developed for homogeneous assay of mammalian cells directly in culture medium. These novel vectors are useful for dual measurements where closely similar reporter structures are preferred or where a single reagent addition is desired.

The Chroma-Luc™ Reporter Vectors contain the Chroma-Luc™ genes, which are related to the commonly used firefly luciferase gene but have been engineered to encode luciferases that emit different colors of light.

### Introduction

In the analysis of cell physiology, it is frequently desirable to correlate multiple parameters associated with a particular cellular process. This correlation may be used to reveal relationships between these parameters or to relate the parameters to an internal control. When using genetic reporters to study processes coupled to transcriptional regulation, multiple parameters can be measured by any of several means involving co-reporters. One popular method uses the Dual-Luciferase® Reporter Assay System<sup>(a,b,c)</sup> (Cat.# E1910), which allows measurement of both the firefly and *Renilla* luciferases from a single sample. The luminescence of these two luciferases is differentiated by their divergent enzymological properties. However, it may be preferable in some applications to use genetic reporters that are more closely related in their structural and chemical properties.

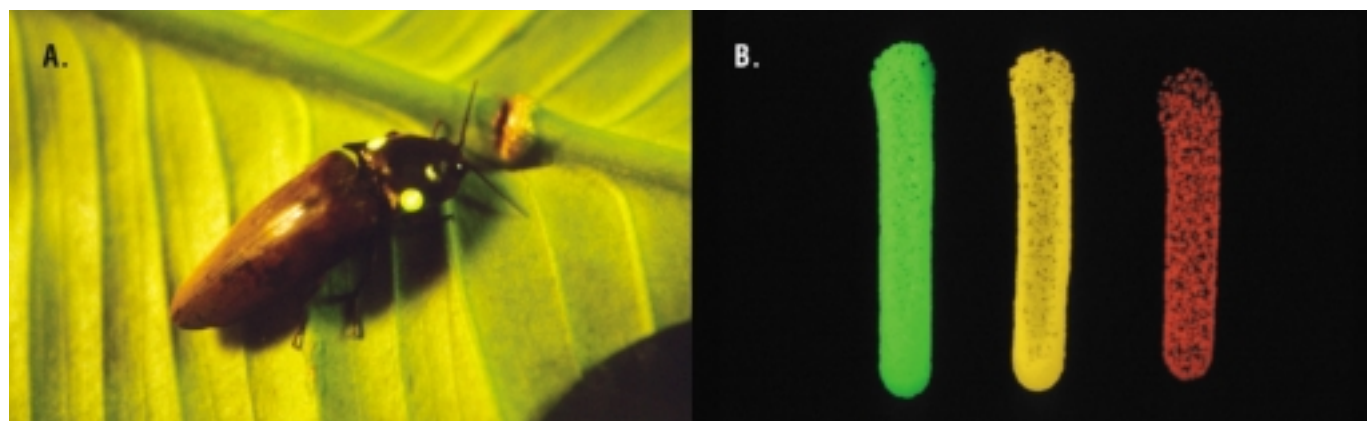
For this purpose we have developed the Chroma-Luc™ Reporter Vectors<sup>(a,d,e,f)</sup>. These vectors contain the Chroma-Luc™ genes, which are related to the commonly used firefly luciferase gene, but have been engineered to encode luciferases that emit different colors of light. Specifically, the *CBG68luc* and *CBG99luc* genes encode green-emitting luciferases, and the *CBRluc* gene encodes a red-emitting luciferase. Both luminescent colors are generated by the same catalytic mechanism as firefly luciferase, so that only a single reagent is needed for these reporters. This is in contrast to the Dual-Luciferase® Reporter Assay, where a separate reagent is added for each of the firefly and *Renilla* luminescent reactions.

Although sharing a common catalytic mechanism with firefly luciferase, the reaction conditions and kinetics of these luciferases are somewhat different. To optimize performance of the Chroma-Luc™ Reporter Vectors, we have also developed the Chroma-Glo™ Reagent<sup>(a,b,e)</sup> (Cat.# E4910, E4920 and E4950). This reagent was designed to provide a homogeneous assay that works directly in mammalian cell culture. The reagent lyses the cells and activates a stable luminescent signal with a half-life greater than 30 minutes. The stability of the luminescence makes the assay suitable for quantitation in plate-reading luminometers lacking integrated reagent injectors. As the green and red luminescent reactions are initiated simultaneously, the activities of the separate luciferases are differentiated with the use of color filters.

### Designed from Luminous Click Beetles

The Chroma-Luc™ genes were developed from naturally occurring luciferase genes of a luminous click beetle, *Pyrophorus plagiophthalmus* (Figure 1, Panel A). This is a large beetle indigenous to the Caribbean, which has two sets of light organs emitting different colors of luminescence; a pair located on the head emits generally green luminescence, and a single organ located in the ventral cleft of the abdomen emits generally yellow-orange luminescence. This particular luminous beetle is unusual in that individual specimens can differ in the color of light emitted from either set of light organs. Complementary DNAs cloned from the ventral light organ encode four luciferases capable of emitting luminescence ranging from green to orange (544 to 593nm). As the encoded luciferases were found to be 95–99% identical in sequence, only a few of the amino acids could be responsible for the color variation.

Further study of these naturally occurring luciferases not only revealed the structural basis for the color variation, but also allowed us to extend the range of luminescence to red (Figure 1, Panel B). One of the cloned luciferases emitting yellow-green luminescence was chosen, because of its brightness, to serve as the template for creating a set of new reporter genes. As with the firefly reporter *luc+*, the peroxisomal targeting sequence of the click beetle luciferase was deleted to retain the reporter within the cytosol of mammalian cells. Limited mutagenesis was performed to increase the thermostability of the luciferase to about 40°C, thereby providing optimum compatibility with mammalian cell culture conditions. Specific amino acids in this luciferase were then substituted to yield both green- and red-emitting



**Figure 1. *Pyrophorus* sp. click beetle and different luminescent colors from the cloned luciferase genes. Panel A.** The pair of light organs on the head of the click beetle, which emit a green luminescence, are easily viewed in this photograph. The light organ on the ventral cleft of the abdomen, which emits a yellow-orange luminescence, only luminesces during flight. **Panel B** (left to right). Green and orange luminescence from genes cloned from the click beetle; red luminescence generated from a mutant luciferase gene.

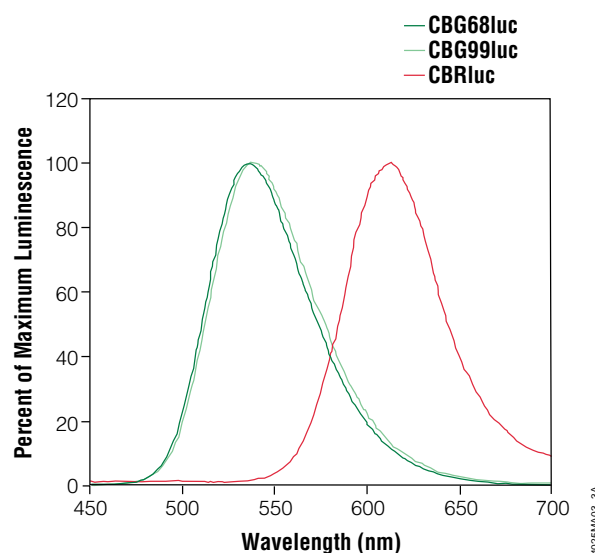
reporters. Additional mutagenesis was performed on the red-emitting luciferase to further optimize the luminescence brightness. The resulting reporters emit peak luminescence separated by >75nm, at approximately 537nm for the green-emitting luciferases and 613nm for the red-emitting luciferase (Figure 2).

## Efficiency from Synthetic Genes

To maximize expression, the genes encoding the luciferases were synthesized using codons preferred in mammalian cells and a Kozak sequence at the initiation codon. Furthermore, the sequences were designed to be essentially devoid of consensus binding sites for mammalian transcription factors. This should minimize potentially anomalous expression brought about through spurious interactions of the reporter genes with endogenous regulatory proteins of the host cells.

The *CBG99luc* gene was designed to be 99% identical to the *CBRluc* gene, thus providing two reporters that are virtually identical in structure but emit different colors of light. In applications where genetic crossover between two very similar genes may be a concern, *CBG68luc* was designed to be only 68.9% identical to *CBRluc*. Note however, that although the gene sequences differ significantly, the luciferases encoded by *CBG68luc* and *CBRluc* are still 98% identical in their amino acid sequences. Each of the reporter genes is provided in a general purpose reporter vector based on the design of the pGL3 Reporter Vectors<sup>(d,b)</sup> and in a control vector containing an SV40 promoter and SV40 enhancer.

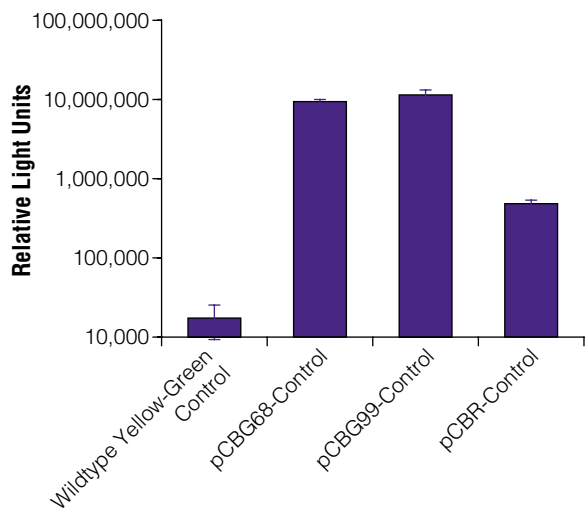
Expression of the synthetic reporter genes in mammalian cells is substantially greater than expression of the wildtype yellow-green luciferase (Figure 3). Expression of the *CBG99luc* and *CBG68luc* genes from the SV40 promoter yielded 660- and 550-fold greater luminescence over the yellow-green luciferase gene, respectively. Expression of the *CBRluc* gene from this promoter yielded 28-fold increase over the yellow-green luciferase gene. However, because this data was not



**Figure 2. Emission spectra of the Chroma-Luc™ enzymes.** Independent populations of CHO cells were transfected with either pCBR-Control, pCBG68-Control, or pCBG99-Control. Twenty-four hours after transfection, the cells were lysed by adding Glo Lysis Buffer (Cat.# E2261). Equal volumes of cell lysate and Chroma-Glo™ Reagent were mixed and incubated for 5 minutes at room temperature. The emission spectra were generated using the Spex Fluorolog<sup>®</sup>-2 spectrofluorometer with the excitation source turned off. Emission data were collected from 450–700nm.

corrected for the spectral efficiency of photon detection by the luminometer, the red-emitting reporter will appear relatively dim compared to the green-emitting reporters or firefly luciferase. Most luminometers use photomultiplier tubes with poor sensitivity in the red region. This bias is not evident in solid-state photon detectors, such as CCD-based luminometers.

In addition to greater expression efficiency, the synthetic genes provide more sensitivity as genetic reporters (Figure 4). Sensitivity is evidenced by the ability to measure a promoter response over the basal expression of the reporter vector. Lower basal expression allows for detection of weaker promoters. Using the SV40 promoter



**Figure 3. Increase in expression from the synthetic Chroma-Luc™ genes.** The three synthetic Chroma-Luc™ genes (*CBRluc*, *CBG68luc*, and *CBG99luc*) and the wildtype Yellow-Green luciferase (*YGluc*) gene were independently cloned into the pGL3-Control Vector, replacing the *luc+* present in this vector. At twenty-four hours post-transfection of CHO cells Chroma-Glo™ Reagent was added in an amount equal to the volume of culture medium and cells. Relative light units were detected using a Berthold Centro LB 960 luminometer. All values have been normalized for transfection efficiency.

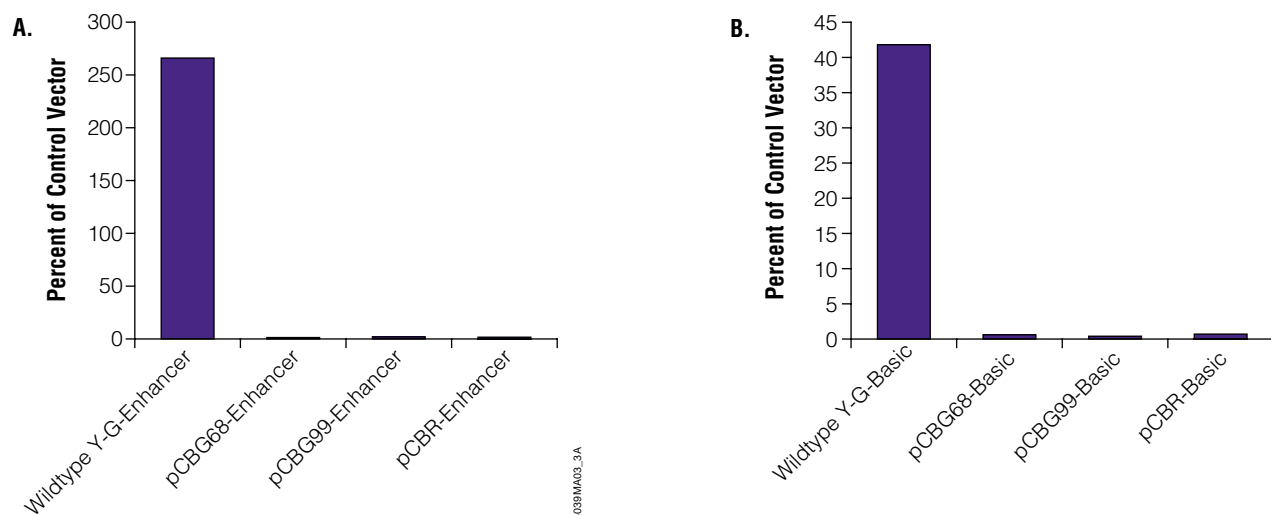
as a reference, the synthetic reporter genes show greater than tenfold improvement in sensitivity. The greatly improved sensitivity may be due in part to removal of anomalous expression effects in the wildtype gene. For example, expression of the wildtype gene in the presence of an enhancer alone is very high, even higher than when combined with the SV40 promoter. Ideally, an enhancer should not activate expression unless promoter elements

are also present. In contrast, expression of the synthetic reporter genes in the presence of the enhancer is quite low, conforming more closely to the anticipated ideal behavior (Figure 4).

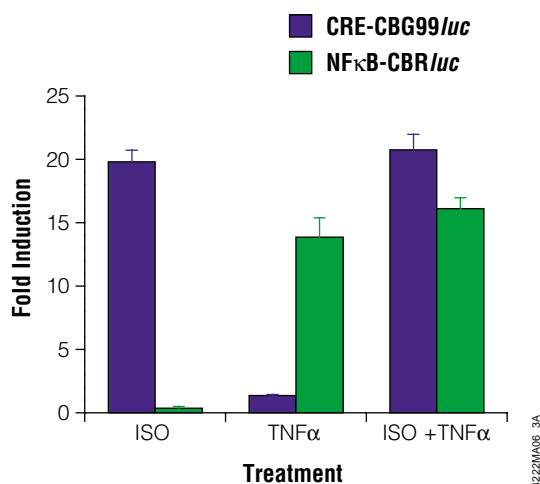
### Independence of Expression

To serve as reliable co-reporters, expression of the green and red luciferase genes must be independent of one another. This was tested using *E. coli*, where intracellular concentration of one luciferase could be varied over 1,000-fold through promoter regulation without affecting expression of the other luciferase (data not shown).

In mammalian cells, we have shown that promoters coupled to different physiological pathways could be independently monitored (Figure 5). The green luciferase gene, *CBG99luc*, was coupled to an NFκB consensus sequence (nuclear factor kappa B), which can be activated by TNFα. On a separate plasmid, the red luciferase gene, *CBRluc*, was coupled to a CRE sequence (cAMP response element), which can be activated by isoproterenol (ISO). Analysis of reporter expression was done on cells co-transfected with both plasmids. The results showed that green luminescence could be specifically activated by addition of TNFα to the culture medium, red luminescence could be specifically activated by addition of ISO to the medium, and both colors could be activated when both factors were added. Moreover, the activation of both reporters together was similar in magnitude to the activation of each reporter separately.



**Figure 4. Reduction in anomalous expression for the synthetic Chroma-Luc™ genes.** The synthetic *CBRluc*, *CBG68luc*, and *CBG99luc* genes and the wildtype *YGluc* (yellow-green luciferase) gene were cloned into a pGL3-Control Vector (containing an SV40 promoter and enhancer), a pGL3-Enhancer Vector (containing only a SV40 enhancer), and pGL3-Basic Vector (no promoter or enhancer added). The vectors were transfected separately into CHO cells. The cells were assayed at 24 hours post-transfection with Chroma-Glo™ Reagent. Relative light units were detected using a Berthold Centro LB 960 luminometer. Expression levels of the Basic and Enhancer vectors are shown as percentages of the corresponding Control vectors. The data show greatly reduced relative expression of the synthetic reporter in the absence of promoter. **Panel A.** Luciferase expression from the pGL3-Enhancer Vector with the synthetic Chroma-Luc™ genes was reduced to an average of 1.8% of control. **Panel B.** The basal level of luciferase transcription from the pGL3-Basic Vector with the synthetic Chroma-Luc™ genes was reduced to an average of 0.6% of control.



**Figure 5. Using the Chroma-Luc™ Technology to simultaneously monitor two promoters.** DNA segments containing either CRE or the NFκB consensus sequence were cloned into pCBG99-Basic (Cat.# E1431) or pCBR-Basic (Cat.# E1411). The resulting constructs, pCRE-CBG99/luc and pNFκB-CBR/luc, were cotransfected into 293 cells. At 24 hours post-transfection the cells received one of three treatments: ISO (1μM)/RO (100μM), TNFα (0.1 μg/ml)/RO (100μM), or ISO (1μM)/RO (100μM) plus TNFα (0.1 μg/ml). Only RO (100μM) was added to the control wells. At six hours after treatment, the cells were harvested and assayed with Chroma-Glo™ Reagent. Relative light units were detected using the Mithras LB 940 (Berthold Technologies) containing a red filter (600 long pass) and a green filter (510/60). The red and green signals were deciphered by using the Chroma-Luc™ Calculator ([www.promega.com/chromacalc/](http://www.promega.com/chromacalc/)). Fold induction was calculated by dividing the luminescence values of the treated samples by the values of the RO control. (RO (RO-0-1724) is a phosphodiesterase (PDE) inhibitor that prolongs the cAMP signal.)

## Conclusion

The Chroma-Luc™ technology consists of red- and green-emitting luciferases and an assay reagent optimized for simultaneous quantification of both colors. This provides a nearly ideal co-reporter system because the reporter enzymes are highly similar in their structures (>98% sequence identity). Furthermore, the DNA and mRNA structures of these reporters are also highly similar when *CBG99luc*, in particular, is coupled with *CBRluc*.

The Chroma-Luc™ genes have been optimized for high expression in mammalian cells, high sensitivity as genetic reporters and low potential for expression artifacts. They have been shown to act independently of one another when co-transfected, allowing independent measurement of separate physiological pathways within a cell. The technology may be useful where differentiable reporters of similar molecular structure are preferred or where only a single reagent addition is desired for both reporters. As the Chroma-Glo™ Reagent is designed for homogeneous assay of the Chroma-Luc™ reporters, it is suitable for rapid quantitation in multiwell plates and high-throughput applications.

## Protocol

- ◆ *Chroma-Luc™ Reporter Vectors Technical Manual*, #TM059 Promega Corporation. ([www.promega.com/tbs/tm059/tm059.html](http://www.promega.com/tbs/tm059/tm059.html))
- ◆ *Chroma-Glo™ Luciferase Assay System Technical Manual*, #TM062 Promega Corporation. ([www.promega.com/tbs/tm062/tm062.html](http://www.promega.com/tbs/tm062/tm062.html))

## Ordering Information

Product	Size	Cat.#
pCBR-Basic Vector <sup>(a,d,e,f)</sup>	20μg	E1411
pCBR-Control Vector <sup>(a,d,e,f)</sup>	20μg	E1421
pCBG68-Basic Vector <sup>(a,d,e,f)</sup>	20μg	E1431
pCBG68-Control Vector <sup>(a,d,e,f)</sup>	20μg	E1441
pCBG99-Basic Vector <sup>(a,d,e,f)</sup>	20μg	E1451
pCBG99-Control Vector <sup>(a,d,e,f)</sup>	20μg	E1461
Chroma-Glo™ Luciferase Assay System <sup>(a,b,e)</sup>	10ml	E4910
	100ml	E4920
	10 × 100ml	E4950

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

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

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

<sup>(d)</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. If any product contains recombinant *Coleoptera* luciferase nucleic acid capable of producing light when expressed, a license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

<sup>(e)</sup> U.S. Pat. Nos. 6,387,675 and 6,552,179, Australian Pat. No. 698424 and other patents pending.

<sup>(f)</sup> For Nonprofit Organization Research Use Only. With respect to research use by for-profit organizations, or any diagnostic or therapeutic uses, please contact Promega Corporation for supply and licensing information.

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

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