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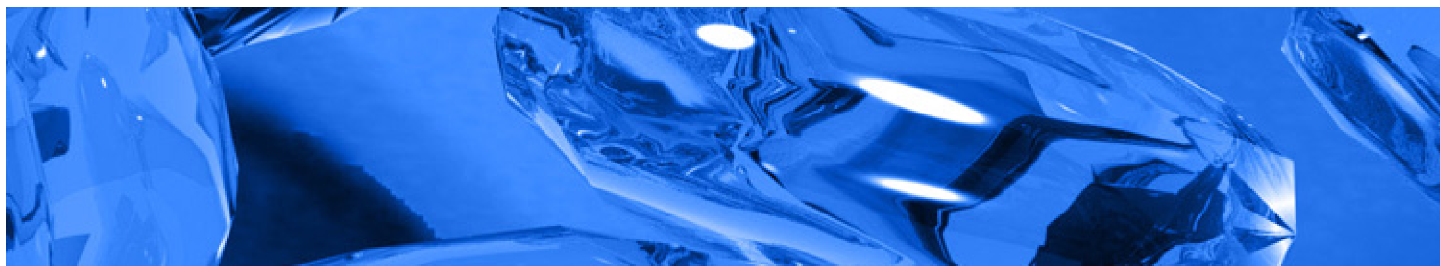


Promega

I. Introduction

Genetic reporter systems have contributed greatly to the study of eukaryotic gene expression and regulation. Although reporter genes have played a significant role in numerous applications, both in vitro and in vivo, they are most frequently used as indicators of transcriptional activity in cells.

Typically, a reporter gene is joined to a promoter sequence in an expression vector that is transferred into cells. Following transfer, cells are assayed for the presence of the reporter by directly measuring the reporter protein itself or the enzymatic activity of the reporter protein. An ideal reporter gene is one that is not endogenously expressed in the cell of interest and is amenable to assays that are sensitive, quantitative, rapid, easy, reproducible and safe.

Analysis of *cis*-acting transcriptional elements is a frequent application for reporter genes. Reporter vectors allow functional identification and characterization of promoter and enhancer elements because expression of the reporter protein correlates with transcriptional activity of the reporter gene. For these types of studies, promoter regions are cloned upstream or downstream of the gene. The promoter-reporter gene fusion is introduced into cultured cells by standard transfection methods or into a germ cell to produce transgenic organisms. The use of reporter gene technology allows characterization of promoter and enhancer elements that regulate cell-, tissue- and development-defined gene expression.

Trans-acting factors can be assayed by co-transfer of the promoter-reporter gene fusion DNA with another cloned DNA expressing a *trans*-acting protein or RNA of interest or by activation of the *trans*-acting factors through treatment of the samples. The protein could be a transcription factor that binds to the promoter region of interest cloned upstream of the reporter gene. For example, when tat protein is expressed from one vector in a transfected cell, the activity of different HIV-1 LTR sequences linked to a reporter gene increases, and the activity increase is reflected in an increase of reporter gene protein activity.

In addition to assessing promoter activity, reporters are commonly used to study the effect of untranslated regions (UTRs) on mRNA stability, protein localization and translation efficiency. For example, microRNA (miRNA) target sites can be inserted downstream or 3′ of the reporter gene to study how microRNAs affect RNA stability.

Reporter proteins can be assayed by detecting inherent characteristics, such as enzymatic activity or spectrophotometric characteristics, or indirectly with antibody-based assays. In general, enzymatic assays are more sensitive due to the small amount of reporter enzyme required to generate detectable levels of reaction products. A potential limitation of enzymatic assays is high background if there is endogenous enzymatic activity in the cell (e.g., β-galactosidase). Antibody-based assays are generally less sensitive but will detect the reporter protein whether it is enzymatically active or not.

Fundamentally, a reporter assay is a means to translate a biomolecular effect into an observable parameter. While there are theoretically many strategies by which this can be achieved, in practice the reporter assays capable of delivering the speed, accuracy and sensitivity necessary for effective screening are based on photon production.

A. Luminescence versus Fluorescence

Photon production is realized primarily through chemiluminescence and fluorescence. Both processes yield photons as a consequence of energy transitions from excited-state molecular orbitals to lower energy orbitals. However, they differ in how the excited-state orbitals are created. In chemiluminescence, the excited states are the product of exothermic chemical reactions, whereas in fluorescence the excited states are created by absorption of light.

This difference in how the excited states are created greatly affects the character of the photometric assay. For instance, fluorescence-based assays tend to be much brighter because high-intensity light can be used to generate the excited state. In chemiluminescent assays, the chemical reactions required to generate excited states usually yield lower rates of photon emission. The greater brightness of fluorescence would appear to correlate with better assay sensitivity, but commonly this is not the case. Assay sensitivity is determined by a statistical analysis of signal relative to background, where the relative signal represents a sample measurement minus the background measurement. Fluorescent assays tend to have much higher backgrounds, leading to lower relative signals.

Fluorescent assays have higher backgrounds primarily because fluorometers cannot discriminate perfectly between the very high influx of photons into the sample and the much smaller emission of photons from the analytical fluorophores. This discrimination is accomplished largely by optical filtration—emitted photons have longer wavelengths than excitation photons—and by geometry because emitted photons typically travel a different path than excitation photons. However, optical filters are not perfect in their ability to differentiate between wavelengths, and photons can change directions through scattering. Chemiluminescence has the advantage that, because photons are not required to create the excited states, they do not constitute an inherent background when measuring photon efflux from a sample. The resulting low background permits precise measurement of very small changes in light.

Fluorescent assays also can be limited by interfering fluorophores within the samples. This is especially problematic in biological samples, which can be replete with a variety of heterocyclic compounds that fluoresce, typically in concentrations much above the analytical fluorophores of interest. The problem is minimized in simple samples, such as purified proteins, but for drug discovery, living cells are increasingly used in high-throughput screening. Unfortunately, cells are enormously complex in their chemical constitutions and can exhibit substantial inherent fluorescence. Screening

compound libraries also is inherently complex. Although each sample may contain only one or a few compounds, the data set from which the drug leads are sifted is cumulated from many thousands of compounds. These compounds also may present problems with fluorescence interference because drug-like molecules typically have heterocyclic structures.

For image analysis of microscopic structure, fluorescence is almost universally preferred over chemiluminescence. Brightness counts because the optics required to image cellular structures are relatively inefficient at light gathering. Thus the low background inherent in chemiluminescence is of little advantage because the signal is usually far below the detection capabilities of imaging devices. Furthermore, imaging is largely a matter of edge detection, which has different signal-to-noise characteristics than simply detecting an analyte. Edge detection relies heavily on signal strength and suffers less from uniform background noise.

In macroscopic measurements (such as in a plate well), which require accurate quantification with high sensitivity, chemiluminescent assays often outperform analogous fluorescence-based assays. Macroscopic measurements are the foundation for most high-throughput screening, which relies heavily on the use of multiwell plates, typically with 96, 384 or 1536 wells, to measure a single parameter in a large number of samples as quickly as possible. Assays based on fluorescence or chemiluminescence can provide high sample throughput. However, fluorescence is more likely to be hindered by light contamination (from the excitation beam) or the chemical compositions of samples and compound libraries. The use of chemiluminescence in high-throughput screening has been limited largely by the lack of available assay methods. Due to its long history, fluorescence has been more commonly used. But new capabilities in chemiluminescence, particularly in bioluminescence, are now allowing new bioluminescent techniques for high-throughput screening.

B. Bioluminescent Reporters

In nature, achieving efficient chemiluminescence is not a trivial matter, as evidenced by the lack of this phenomenon in daily life. The large energy transitions required for visible luminescence generally are disfavored over smaller ones that dissipate energy as heat, normally through interactions with surrounding molecules, especially water molecules in aqueous solutions. Because energy can be lost through these interactions, chemiluminescence depends strongly on environmental conditions. Thus, chemiluminescent assays often incorporate hydrophobic compounds such as micelles to protect the excited state from water or rely on energy transfer to fluorophores that are less sensitive to solvent quenching. Another difficulty with chemiluminescence is efficient coupling of the reaction to the creation of excited-state orbitals. While chemiluminescence has relied on the ingenuity of chemists, bioluminescence, a form of chemiluminescence, has relied more on the processes of natural evolution. As luminous organisms through the eons were selected by the brightness of their light, their light-producing enzymes (i.e., luciferases) have evolved both to maximize chemical couplings to generate the excited states and to protect the excited states from water.

Although most people are aware of bioluminescence primarily through the nighttime displays of fireflies, there are many distinct classes of bioluminescence derived through separate evolutionary histories. These classes are widely divergent in their chemical properties, yet they all undergo similar chemical reactions. The classes are all based on the interaction of the enzyme luciferase with a luminogenic substrate to produce light (Figure 8.1). The luciferases that are used most widely in high-throughput screening are beetle luciferases (including firefly luciferase), *Renilla* luciferase and a modified deep sea shrimp luciferase (NanoLuc® luciferase). Beetle luciferases are the most common of this group and are used in a variety of reporter applications. *Renilla* and NanoLuc® luciferases are used primarily for reporter gene applications, although their uses are expanding. Click beetle luciferases are becoming better known and offer a range of luminescence color options. Although not considered a luciferase enzyme, the photoprotein aequorin also is used as a luminescent reporter almost exclusively to monitor intracellular calcium concentrations.

Intracellular luciferase is typically quantified by adding a buffered solution containing detergent to lyse the cells and a luciferase substrate to initiate the luminescent reaction. Luminescence will slowly decay due to side reactions, causing irreversible inactivation of the enzyme. The nature of these side reactions is not well understood, but they are probably due to formation of damaging free radicals. To maintain steady luminescence of firefly and *Renilla* luciferase assays over an extended period of time, ranging from minutes to hours, it is often necessary to inhibit the luminescent reaction to various degrees. This inhibition can reduce the rate of luminescence decay to the point where the reduction in signal is insignificant over the time required to measure multiple samples. Even under these conditions, as few as 10^{-20} moles of luciferase or less per sample may be quantified. This corresponds to roughly 10 molecules per cell.

Unlike firefly and *Renilla* luciferases, NanoLuc® luciferase does not require enzyme inhibition for extended half-life assays. NanoLuc®-based assays, which use a novel furimazine substrate to measure NanoLuc® luciferase activity, exhibit a much brighter signal and a much slower rate of luminescence decay, with a signal half-life of approximately 120 minutes. These extended half-life assays are convenient for reporter gene applications because sample processing is not required prior to reagent addition. Simply add the reagent, and measure the resulting luminescence.

Most reporter gene assays involve either one reporter gene or two. Most often, the second reporter is expressed from a "control" vector to normalize results of the experimental reporter. For example, the second reporter can control for variation in cell number or transfection efficiency. Typically, the control reporter gene is driven by a constitutive promoter, and the control vector is cotransfected with the "experimental" vector. Different reporter genes are used for the control and experimental vectors so that the relative activities of the two reporter products can be assayed individually.

Alternatively, you can design dual-reporter bioluminescence assays in which both reporter genes are used as experimental reporters. Such dual-reporter assays can be particularly useful for efficiently extracting more information in a single experiment. For more information, see the Special Considerations for High-Throughput Assays section.

The Introduction to Reporter Gene Assays animation demonstrates the basic design of a reporter assay using the Dual-Luciferase® Reporter Assay System as an example to study promoter structure, gene regulation and signaling pathways.

C. Applications

Basic research into bioluminescence has led to practical applications, particularly in molecular biology, where bioluminescent enzymes are widely used as genetic reporters. Moreover, the value of these applications has grown considerably over the past decade as the traditional use of reporter genes has broadened to cover wide-ranging aspects of cell physiology.

The conventional use of reporter genes is largely to analyze gene expression and dissect the function of *cis*-acting genetic elements such as promoters and enhancers (so-called "promoter bashing"). In typical experiments, deletions or mutations are made in a promoter region, and their effects on coupled expression of a reporter gene are quantitated. However, the broader aspect of gene expression entails much more than transcription alone, and reporter genes also can be used to study other cellular events, including events that are not related to gene expression.

Some examples of analytical methodologies that use luciferase include:

- Assays and biosensors that monitor cell-signaling pathways. For example, the GloResponse™ Cell Lines facilitate rapid and convenient analysis of cell signaling through the nuclear factor of activated T-cells (NFAT) pathway or cyclic AMP (cAMP) response pathways via activation of a luciferase reporter gene. The GloSensor™ biosensor is a genetically modified form of firefly luciferase into which a cAMP-binding protein moiety has been inserted such that cAMP binding induces a conformational change, leading to increased light output.
- RNA interference to study how double-stranded RNA (dsRNA) suppresses expression of a target protein by stimulating specific degradation of the target mRNA. Luciferase reporters can be used to quantitatively evaluate microRNA activity by inserting miRNA target sites downstream or 3′ of the firefly luciferase gene. For example, the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Cat.# E1330) is based on dual-luciferase technology, with firefly luciferase as the primary reporter to monitor mRNA regulation and *Renilla* luciferase as a control reporter for normalization.
- Identification of interacting pairs of proteins in vivo using a system known as the two-hybrid system (Fields *et al.* 1989). The interacting proteins of interest are brought together as fusion partners—one is fused with a specific DNA-binding domain, and the other protein is fused with a transcriptional activation domain. The physical interaction of the two fusion partners is necessary for functional activation of a reporter gene driven by a basal promoter and the DNA motif recognized by the DNA-binding protein. This system was originally developed with yeast but also is used in mammalian cells.
- Reporters of protein abundance and stability. The rate of protein turnover is tightly regulated for many signaling proteins. Protein stabilization and subsequent accumulation can occur in response to cell signaling events and changing cellular conditions and result in activation of downstream transcriptional events. The NanoLuc® Stability Sensor Vectors (Cat.# N1381 and Cat.# N1391) enable stability studies of two key signaling proteins, HIF1A and NRF2, and provide a method to directly measure the cellular effects of hypoxia and oxidative stress, respectively (Robers *et al.* 2014).
- Bioluminescence resonance energy transfer (BRET) to monitor protein:protein interactions, where two fusion proteins are made: one fused to the bioluminescent *Renilla* or NanoLuc® luciferase and another protein fused to a fluorescent molecule. Interaction of the two fusion proteins results in energy transfer from the bioluminescent molecule to the fluorescent molecule, with a concomitant change from blue light to green light (Angers *et al.* 2000).
- Live-cell and in vivo imaging. Luciferase genes can be used as light-emitting reporters in cellular and animal models. Visualization of reporter expression using live-cell luciferase substrates or secreted forms of luciferase allows nondestructive, quantitative assays and multiple measures of the same samples without perturbation.

For more information, view the Introduction to Bioluminescent Assays animation.

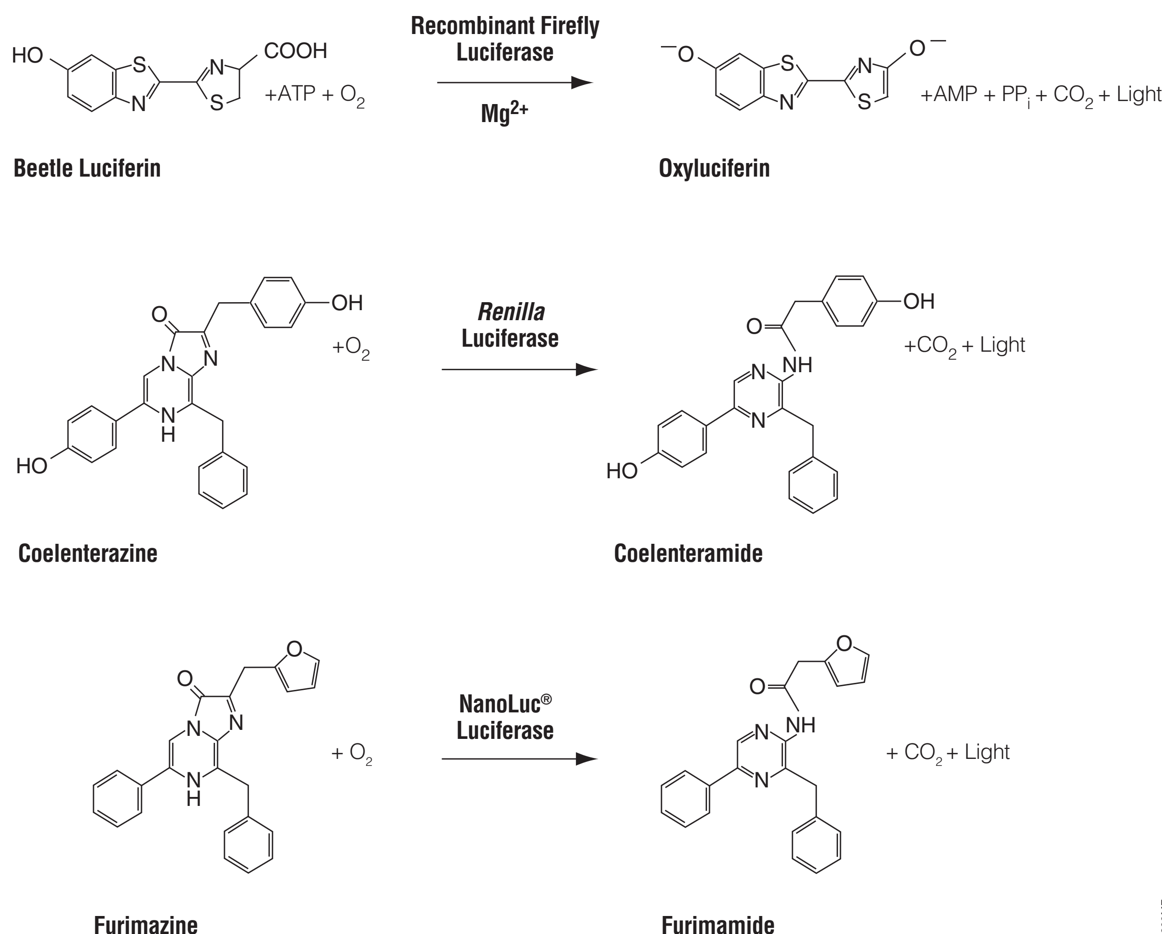


Figure 8.1. Diagram of firefly, *Renilla* and NanoLuc® luciferase reactions with their respective substrates (beetle luciferin, coelenterazine and furimazine) to yield light.

II. Luciferase Genes and Vectors

A. Biology and Enzymology

Bioluminescence as a natural phenomenon is widely experienced with amazement at the prospect of living organisms creating their own light. Luciferase genes have been cloned from bacteria, beetles (e.g., firefly and click beetle), *Renilla*, deep sea shrimp (*Oplophorus*), *Aequorea*, *Vargula* and *Gonyaulax* (a dinoflagellate). Of these, only luciferases from bacteria, beetles, deep sea shrimp and *Renilla* have found general use as indicators of gene expression. Bacterial luciferase is generally used to provide autonomous luminescence in bacterial systems through expression of the lux operon but is not often used for analysis in mammalian cells.

Firefly Luciferase

Firefly luciferase is by far the most commonly used bioluminescent reporter. This monomeric enzyme of 61kDa was cloned from the North American firefly (*Photinus pyralis*) and catalyzes a two-step reaction in which oxidation of D-luciferin yields light, usually in the green to yellow region, typically 550–570nm (Figure 8.1). The first step is activation of luciferin with ATP to give a luciferyl-adenylate and pyrophosphate. In the second step, the luciferyl-adenylate reacts with molecular oxygen to yield oxyluciferin in an electronically excited state and CO_2. The excited-state oxyluciferin then returns to the ground state with concomitant release of light. Upon mixing with substrates, firefly luciferase produces an initial burst of light that decays over about 15 seconds to a low level of sustained luminescence. This kinetic profile reflects the accumulation of one or more potent luciferase inhibitors, such as dehydroluciferyl-AMP, thus limiting catalytic turnover.

Various strategies have been tried to generate stable luminescence and make the assay more convenient for routine laboratory use. The most successful of these incorporates coenzyme A (CoA) to yield maximal luminescence intensity that slowly decays over several minutes. CoA appears to help clear the active site of potent inhibitors like dehydroluciferyl-AMP, thus sustaining luminescence over longer periods of time. An optimized assay containing coenzyme A generates relatively stable luminescence in less than 0.3 seconds, with linearity over a 100-millionfold range of enzyme concentrations. The assay sensitivity allows quantitation of fewer than 10^{-20} moles of enzyme.

The popularity of native firefly luciferase as a genetic reporter is due to the sensitivity and convenience of the enzyme assay and tight coupling of protein synthesis with enzyme activity. Firefly luciferase, which is encoded by the *luc* gene, is a monomer that does not require any post-translational modifications; it is available as a mature enzyme directly upon translation of its mRNA. Catalytic competence is attained immediately after release from the ribosome. Also, firefly luciferase has a relatively short half-life in cells compared to other commonly used reporters.

NanoLuc® Luciferase

NanoLuc® luciferase is a small monomeric enzyme (19.1kDa, 171 amino acids) based on the luciferase from the deep sea shrimp *Oplophorus gracilirostris* (Hall *et al.* 2012). This engineered enzyme uses a novel substrate, furimazine, to produce high-intensity, glow-type luminescence (λmax = 465nM) in an ATP-independent reaction (Figure 8.1). The signal half-life is >2 hours, and luminescence is about 100-fold brighter than that of firefly or *Renilla* luciferase (Figure 8.2). See why the brightness of NanoLuc® Luciferase matters.

In mammalian cells, NanoLuc® luciferase shows no evidence of post-translational modifications, disulfide bonds or subcellular partitioning. The enzyme is active over a broad pH range (pH 6–8), exhibits high physical stability and retains activity at temperatures of up to 55°C or in culture medium for >15 hours at 37°C.

Unlike other forms of luciferase, NanoLuc® luciferase is ideally suited for both standard (lytic) and secretion-based (nonlytic) reporter gene applications. The small size of the gene (513bp) and encoded protein is ideal for viral applications and protein fusions.

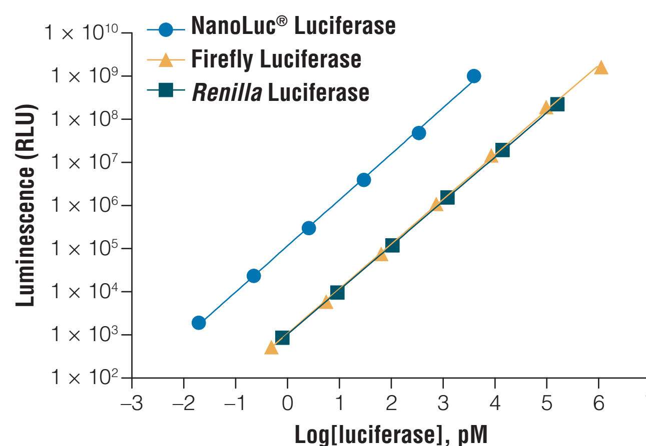


Figure 8.2. A comparison of the sensitivity of NanoLuc®, firefly and *Renilla* luciferase assays. Luminescence was measured from varying concentrations of purified luciferases after mixing the reporter enzyme with its respective detection reagent. NanoLuc® luciferase was approximately 100-fold brighter than firefly or *Renilla* luciferases at equivalent concentrations. Luminescence was measured using the Nano-Glo® Luciferase Assay for NanoLuc® luciferase, ONE-Glo™ Luciferase Assay System for firefly luciferase and *Renilla*-Glo™ Luciferase Assay System for *Renilla* luciferase.

***Renilla* Luciferase**

Renilla luciferase is a 36kDa monomeric enzyme that catalyzes the oxidation of coelenterazine to yield coelenteramide and blue light of 480nm (Figure 8.1). The host organism, *Renilla reniformis* (sea pansy), is a coelenterate that creates bright green flashes upon tactile stimulation, apparently to ward off potential predators. The green light is created through association of the luciferase with a green fluorescent protein and represents a natural example of BRET.

Although *Renilla* and *Aequorea* are both luminous coelenterates based on coelenterazine oxidation and both have a green fluorescent protein, their respective luciferases are structurally unrelated. In particular, *Renilla* luciferase does not require calcium in the luminescent reaction. As a reporter molecule, *Renilla* luciferase, which is encoded by the *Rluc* gene, provides many of the same benefits as firefly luciferase. Historically, the presence of nonenzymatic luminescence, termed autoluminescence, reduced assay sensitivity; however, improvements in assay chemistry have nearly eliminated this problem. In addition, the simplicity of the *Renilla* luciferase chemistry and, more recently, improvements to the luciferase substrate have enabled quantitation of *Renilla* luciferase from living cells in situ or in vivo.

Click Beetle Luciferase

Click beetle and firefly luciferase belong to the same beetle luciferase family. Hence, the size and enzymatic mechanism of click beetle luciferase are similar to those of firefly luciferase. What makes the click beetle unique is the variety of luminescence colors it emits. Genes cloned from the ventral light organ of a luminous click beetle, *Pyrophorus plagiophthalamus*, encode four luciferases capable of emitting

luminescence ranging from green to orange (544–593nm). The Chroma-Luc™ luciferases were developed from these naturally occurring luciferase genes to generate luminescence colors as different as possible: a red luciferase (611nm) and two green luciferases (544nm each). These luciferase genes were codon-optimized for mammalian cells and are nearly identical to one another, with a maximum of 8 amino acids difference between any two of these genes. The two green luciferase genes generate very similar luciferase proteins; however, one is maximally similar (~98%) to the DNA sequence for the red luciferase, while the other is divergent (~68%). Therefore, experimental and control reporter genes and proteins within an experiment can be almost identical. Under circumstances where genetic recombination is a concern, the divergent luciferase gene pair may be useful.

B. Gene Optimization

An ideal genetic reporter should: i) express uniformly and optimally in the host cells; ii) only generate responses to effectors that the assay intends to monitor (avoid anomalous expression); and iii) have a low intrinsic stability to quickly reflect transcriptional dynamics. Despite their biology and enzymology, native luciferases are not necessarily optimal as genetic reporters. In the past decade, Promega scientists have made significant improvements in luciferase expression, reducing the risk of anomalous expression and destabilizing these reporters. The key strategies to achieve these improvements are described here.

Peroxisomal Targeting Site Removal
Normally, in the firefly light organ, luciferase is located in specialized peroxisomes of photocytic cells. When the firefly or click beetle enzyme is expressed in foreign hosts, a conserved translocation signal causes luciferase to accumulate in peroxisomes and glyoxysomes. Peroxisomal and glyoxysomal localization of luciferase may interfere with normal cellular physiology and performance of the genetic reporter. For instance, luciferase accumulation in the cell might be differentially affected if the enzyme is distributed into two different subcellular compartments.

The peroxisomal translocation signal in firefly and click beetle luciferases has been identified as the C-terminal tripeptide sequence, -Ser-Lys-Leu. Removal of this sequence abolishes import into peroxisomes. To develop an optimal cytoplasmic form of the luciferase gene, Promega scientists replaced the existing C-terminal sequence with a new C-terminal sequence, -Ile-Ala-Val. Consistently, this modified luciferase yielded about 4- to 5-fold greater luminescence than the native enzyme when expressed in NIH/3T3 cells. *Renilla* and NanoLuc® luciferases do not contain a targeting sequence and are not affected by peroxisomal targeting.

Codon Optimization
Although redundancy in the genetic code allows amino acids to be encoded by multiple codons, different organisms favor some codons over others. The efficiency of protein translation in a non-native host cell can be increased substantially by adjusting the codon usage frequency while maintaining the same gene product. The native luciferase genes cloned from beetles (firefly or click beetle), sea pansy (*Renilla reniformis*) and deep sea shrimp use codons that are not optimal for expression in mammalian cells. Therefore, Promega scientists systematically altered the codons to the preferred ones while removing inappropriate or unintended transcription regulatory sequences used in mammalian cells. As a result, a significant increase in luciferase expression levels was achieved, up to several hundredfold in some cases (Figures 8.3 and 8.4).

Cryptic Regulatory Sequence Removal
The presence of cryptic regulatory sequences in a reporter gene may adversely affect transcription, resulting in anomalous expression of the reporter gene. Removal of these sequences reduces the risk of anomalous expression. A cryptic regulatory sequence can be a transcription factor-binding site or a promoter module (defined as at least two transcription factor-binding sites separated by a spacer; Klingenhoff *et al.* 1999). Additionally, it is not uncommon for an enhancer element to elevate levels of transcription in the absence of a promoter sequence or increase basal levels of gene expression in the presence of transcription regulatory sequences. These regulatory sequences can exhibit synergistic or antagonistic functions (Klingenhoff *et al.* 1999).

These cryptic regulatory sequences in the *luc* gene were removed without changing the amino acid sequence to create the *luc2* gene. In addition, sequences resembling splice sites, poly(A) addition sequences, Kozak sequences (translation start sites for mammalian cells), *E. coli* promoters or *E. coli* ribosome-binding sites were removed wherever possible. This process has led to a greatly reduced number of cryptic regulatory sequences (Figure 8.5) and therefore a reduced risk of anomalous transcription. A similar process was performed using *Renilla* luciferase to produce the *hRluc* gene.

Degradation Signal Addition
When performing reporter assays, the total accumulated reporter protein within cells is measured. This accumulation occurs over the intracellular lifetime of the reporter, which is determined by both protein and mRNA stability. If transcription is changing during this lifetime, then the resulting accumulation of reporter will reflect a collection of different transcriptional rates. The longer the lifetime of the reporter protein, the greater the collection of different transcriptional rates pooled into the reporter assay. This pooling process has a "dampening effect" on the apparent transcriptional dynamics, making changes in the transcriptional rate more difficult to detect. This can be remedied by reducing the reporter lifetime, thus reducing the pooling of different transcriptional rates into each reporter measurement. The resulting improvement in reporter dynamics is applicable to both upregulation and downregulation of gene expression.

Ideally, the reporter lifetime would be reduced to zero, completely eliminating the pooling of different transcriptional rates in each assay measurement. Only the

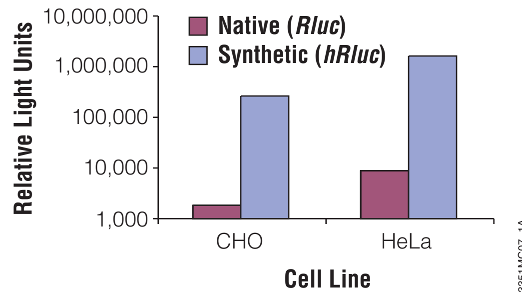


Figure 8.3. The synthetic *Renilla* luciferase gene supports higher expression than the native gene in mammalian cells. CHO and HeLa cells were transfected with the pGL3-Control Vector (Cat.# E1741; contains the SV40 enhancer/promoter) harboring either the synthetic *hRluc* or native *Rluc* gene. Cells were harvested 24 hours after transfection and *Renilla* luciferase activity assayed using the Dual-Luciferase® Reporter Assay System.

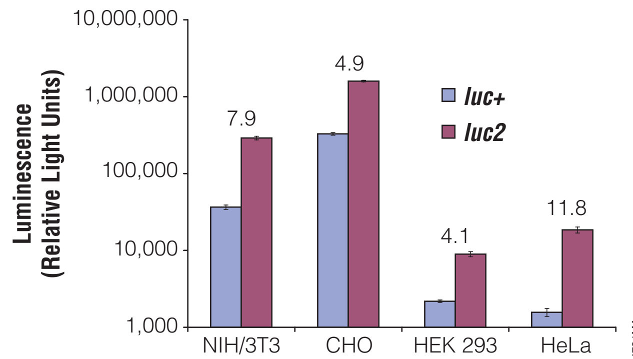


Figure 8.4. The firefly *luc2* gene displays higher expression than the *luc+* gene. The *luc2* gene was cloned into the pGL3-Control Vector, replacing the *luc+* gene. Thus both firefly luciferase genes were in the same pGL3-Control Vector backbone. The two vectors containing the firefly luciferase genes were co-transfected into NIH/3T3, CHO, HEK 293 and HeLa cells using the phRL-TK Vector as a transfection control. Twenty-four hours post-transfection the cells were lysed with Passive Lysis 5X Buffer (Cat.# E1941), and luminescence was measured using the Dual-Luciferase® Reporter Assay System (Cat.# E1910). Firefly luciferase luminescence (in relative light units) was normalized to *Renilla* luciferase expression from the phRL-TK Vector. The increase in expression of *luc2* compared to *luc+*, indicated as the fold increase, for each cell line is listed above each pair of bars. A repeat of this experiment yielded similar results.

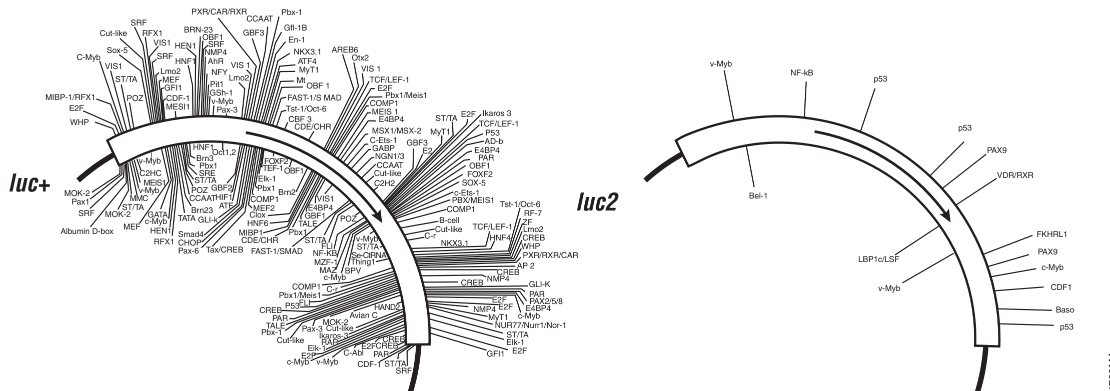


Figure 8.5. Reduced number of consensus transcription factor-binding sites for the *luc2* gene. The number of consensus transcription factor-binding sites identified in the *luc+* gene is greatly reduced in the *luc2* gene.

transcription rate at the instant of the assay would be represented by reporter protein accumulation within the cells. Unfortunately, a zero lifetime also would yield zero accumulation, and thus no reporter could be measured. A compromise must be reached since, as reporter lifetime decreases, so does the amount of reporter available for detection. This is where the high sensitivity of luminescent assays is useful. Relative to other reporter technologies, the intracellular stability of luciferase reporters may be greatly reduced while still providing a signal well above background. Thus, the high sensitivity of luciferase assays permits greater dynamics of the luciferase reporters.

NanoLuc® luciferase is more stable than beetle and *Renilla* luciferase reporter proteins. Due to protein stability, reporter response may lag behind the underlying

transcriptional events by several hours. To reduce the cellular half-life of the reporter gene and improve reporter responsiveness, Promega scientists developed destabilized luciferase reporters by genetically fusing a protein degradation sequence to the luciferase gene products (Li *et al.* 1998). After evaluating many degradation sequences for their effect on response rate and signal magnitude, Promega scientists chose two sequences: the PEST protein degradation sequence and a second sequence composed of two protein degradation sequences (CL1 and PEST). The *luc2* gene with a PEST sequence (*luc2P*) and CL1 and PEST sequences (*luc2CP*), named the RapidResponse™ genes, respond more rapidly to stimuli but at the expense of signal intensity. The *luc2P* gene (approximately 1-hour protein half-life; half-life varies by cell line) responds much more rapidly than *luc2* (approximately 3-hour half-life), with moderate signal intensity. The *luc2CP* gene (approximately 0.4-hour half-life) responds more quickly and has lowest signal intensity. The NanoLuc® gene product with a PEST sequence (*NlucP*) has a protein half-life of about 20 minutes but still maintains a bright signal due to the enzyme's higher specific activity. These destabilized reporters respond faster and often display greater signal-to-background compared to nondestabilized counterparts.

Addition of a Secretion Signal

Native *Oplophorus* luciferase catalyzes the luminescent oxidation of the substrate coelenterazine to produce blue light. The enzyme is secreted by the shrimp as a defense mechanism against predation. In the laboratory, such a secreted reporter is useful when extracellular detection is preferred—for example, to avoid cell lysis and maintain cell viability. To maximize the efficiency of protein secretion in mammalian cells, Promega scientists replaced the native secretion signal with a mammalian protein secretion signal, resulting in the *secNluc* gene. Secreted NanoLuc® luciferase is ideal for nondestructive reporter gene assays in cultured cells due to the enzyme's thermostability, which allows a stable enzyme to accumulate in the extracellular medium.

C. pGL4 Luciferase Reporter Vectors

Vectors used to deliver the reporter gene to host cells are critical for overall reporter assay performance. Cryptic regulatory sequences such as transcription factor-binding sites and promoter modules within the vector backbone can lead to high background and anomalous responses. This is a common issue for mammalian reporter vectors, including the pGL3 Luciferase Reporter Vectors. Promega scientists extended the successful "cleaning" strategy described above for reporter genes to the entire pGL3 Vector backbone, removing cryptic regulatory sequences wherever possible, while maintaining reporter functionality. Other modifications include a redesigned multiple cloning region with a unique SfiI site to facilitate easy transfer of the DNA element of interest, removal of the f1 origin of replication and deletion of an intron. In addition, a synthetic poly(A) signal/transcriptional pause site was placed upstream of either the multiple cloning region (in promoterless vectors) or HSV-TK, CMV or SV40 promoter (in promoter-containing vectors). This extensive effort resulted in the totally redesigned and unique vector backbone of the pGL4 Vectors.

The pGL4 family of luciferase vectors incorporates a variety of features such as your choice of firefly or *Renilla* luciferase, Rapid Response™ versions for improved temporal response, mammalian selectable markers, basic vectors without promoters, promoter-containing control vectors and predesigned vectors with your choice of several response elements (Figure 8.6). These vectors encode optimized reporter genes that offer additional luminescence colors, improved codon usage for mammalian expression and fewer cryptic regulatory sequences such as transcription factor-binding sites that could affect protein expression in mammalian cells.

D. pNL Vectors

NanoLuc® luciferase is available in a variety of vectors for use in reporter gene assays of transcriptional control. These pNL vectors are based on the pGL4 vector backbone and thus offer many of the same advantages: Removal of transcription factor-binding sites and other potential regulatory elements to reduce the risk of anomalous results, easy sequence transfer from existing plasmids and a choice of several promoter sequences. The family of vectors offer a choice of NanoLuc® genes (unfused *Nluc*, PEST-destabilized *NlucP* and secreted *secNluc*). These NanoLuc® gene variations are codon-optimized and have had many potential regulatory elements or other undesirable features such as common restriction enzyme sites removed.

III. Luciferase Reporter Assays and Protocols

The challenge when designing bioluminescent assays is harnessing this efficient light-emitting chemistry for analytical methodologies. Most commonly this is done by holding the concentration of each reaction component constant, except for one component that is allowed to vary in relation to the biomolecular process of interest. When the reaction is configured properly, the resultant light is directly proportional to the variable component, thus coupling an observable parameter to the reaction outcome. In assays using luciferase, the variable component may be the luciferase itself, its substrates or cofactors. Due to the very low backgrounds in bioluminescence, the linear range of this proportionality can be enormous, typically extending 10^4- to 10^8-fold over the concentration of the variable component.

The following section provides information about specific bioluminescent reporters and assays, including how to choose the correct reporter genes to suit your research needs. Tables 8.1, 8.2, 8.3 and 8.4 include a summary of available luciferase genes, assays and reagents.

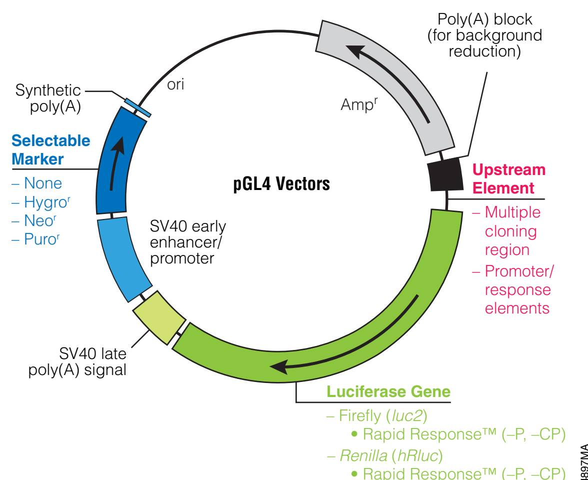


Figure 8.6. The family of pGL4 Luciferase Reporter Vectors incorporates a variety of additional features, such as a choice of luciferase genes, Rapid Response™ versions, a variety of mammalian selectable markers and vectors with or without promoters and response elements.

A. Single-Reporter Assays

Assays based on a single reporter provide the quickest and least expensive means to acquire gene expression data from cells. However, because cells are inherently complex, the information gleaned from a single-reporter assay may be insufficient to achieve detailed and accurate results. Thus, one of the first considerations when choosing a reporter methodology is deciding if the depth of information from a single reporter is sufficient or if you would benefit from the additional information that can be gleaned from a second reporter (e.g., normalization of differences in transfection efficiency or cell number). If more information is required, see the Dual-Reporter Assays section.

When choosing a luciferase assay, a trade-off between luminescence intensity and duration is often necessary because bright reactions fade relatively quickly. Using a firefly or *Renilla* luciferase assay that yields maximum luminescence results in higher sensitivity, but using an assay with a longer signal half-life and a more stable luminescent signal is more convenient when performing assays in multiwell plates. Of the extended half-life firefly luciferase assays, we recommend the ONE-Glo™ Luciferase Assay System (Cat.# E6110) and ONE-Glo™ EX Luciferase Assay System (Cat.# E8110). The ONE-Glo™ Luciferase Assay System yields maximal luminescence intensity, has a 45-minute half-life and is more tolerant of nonoptimal reaction conditions. The ONE-Glo™ EX Reagent has a longer signal half-life (approximately 2 hours), is more stable at 4°C or room temperature once reconstituted, exhibits less interference from phenol red in cell culture medium and has less odor than other luciferase assay reagents.

The Steady-Glo® Luciferase Assay System provides even longer luminescence duration but with lower intensity. The Steady-Glo® Reagent is added directly to the culture medium for mammalian cells, so prior cell lysis is not necessary. This allows you to grow cells in multiwell plates, and then measure reporter expression in a single step. *Renilla* luciferase assays with different signal intensities and half-lives are also available.

Sacrificing luminescence intensity for signal half-life is less of a concern with NanoLuc® luciferase. The Nano-Glo® Luciferase Assay System (Cat.# N1110) provides a simple, single-addition reagent that generates a glow-type signal with a half-life of approximately 120 minutes in commonly used tissue culture media. The reagent contains an integral lysis buffer, allowing direct use with cells expressing NanoLuc® luciferase, but is also compatible with culture medium containing secreted luciferase.

Additional Resources for Single-Reporter Assays

Technical Bulletins and Manuals

TM369	*Nano-Glo® Luciferase Assay System Technical Manual*
TM292	*ONE-Glo™ Luciferase Assay System Technical Manual*
TM432	*ONE-Glo™ EX Luciferase Assay System Technical Manual*
TM052	*Bright-Glo™ Luciferase Assay System Technical Manual*
TM051	*Steady-Glo® Luciferase Assay System Technical Manual*
TB281	*Luciferase Assay System Technical Bulletin*
TM055	*Renilla Luciferase Assay System Technical Manual*
TM329	*Renilla-Glo™ Luciferase Assay System Technical Manual*
TM259	*pGL4 Luciferase Reporter Vectors Technical Manual*

Citations

Emonet , S.F. *et al.* (2009) Generation of recombinant lymphocytic choriomeningitis virus with trisegmented

genomes stably expressing two additional genes of interest. *Proc. Natl. Acad. Sci. USA* **106**, 3473–8.

The lymphocytic choriomeningitis virus (LCMV) was used as a model to create a trisegmented recombinant arenavirus in which viral genes were replaced by a gene of interest. One such engineered virus, r3LCMV CAT/FLuc, was used in a pilot screen to identify anti-arenaviral compounds. Firefly luciferase (FLuc) activity was measured using the ONE-Glo™ Luciferase Assay System.

PubMed Number: 19208813

Cai , Y. *et al.* (2014) DNA transposition by protein transduction of the *piggyBac* transposase from lentiviral Gag precursors. *Nucleic Acids Res.* **42**, e28.

Researchers were looking for alternative methods to using transposase vectors carried by lentiviruses to insert genes into cellular DNA without the cytotoxicity that may occur if the transposase gene integrated into the genome. The authors generated lentiviral particles that carried the transposase protein to deliver genes at an equal efficiency as the conventional plasmid-based method. They cloned the NanoLuc® luciferase reporter gene into a gag-pol-integrase-defective packaging construct and cloned firefly luciferase into the piggyBac transposon lentiviral vector. They also created Gag-pol constructs that expressed a hyperactive piggyBac transposase. Lentiviral particles (LPs) were generated by cotransfection of several plasmids into 293T cells. These NanoLuc® LPs were added to HeLa cells with or without pseudotyping by Vesicular Stomatitis Virus envelope glycoprotein, and after 48 hours, luminescence was measured using the Nano-Glo® Luciferase Assay System. To analyze how well the firefly luciferase gene was transferred, HaCaT and ARPE-19 cells were incubated with increasing amounts of either wildtype or mutated PBase/firefly luciferase transposon LPs. After ten days, the transduced cells were assessed for luminescence using the ONE-Glo™ Luciferase Assay System. HEK293 cells, primary keratinocytes and normal human dermal fibroblasts also were incubated with either wildtype or mutated PBase/firefly luciferase transposon LPs, and after eight days, firefly luminescence was measured.

PubMed Number: 24089552

Lin , P.F. *et al.* (2003) A small molecule HIV-1 inhibitor that targets the HIV-1 envelope and inhibits CD4 receptor binding. *Proc. Natl. Acad. Sci. USA* **100**, 11013–8.

To test the effect of BMS-378806, a new small-molecule inhibitor of HIV-1, a cell fusion assay was developed. Target cells that stably expressed CD4, CXCR4 or CCRS receptors and carried a responsive luciferase plasmid were prepared. Effector cells were transiently transfected with the HIV coat protein gp160 from various strains of virus and a plasmid to activate the response element controlling luciferase expression. If the cells fused, luciferase was synthesized. To measure cell fusion, effector cells were plated with target cells at a ratio of 1:2 in 96-well plates, and then incubated with various concentrations of BMS-378806 for 12–24 hours. Luciferase activity was measured using Steady-Glo® Luciferase Assay System.

PubMed Number: 12930892

Promega Publications

ONE-Glo™ Luciferase Assay System: New substrate, better reagent.

The bioluminescence advantage

pGL4 Vectors: A new generation of luciferase reporter vectors.

Bright-Glo™ and Steady-Glo® Luciferase Assay Systems: Reagents for academic and industrial applications.

B. Dual-Reporter Assays

The most commonly used dual-reporter assays measure both firefly and *Renilla* luciferase activities. These luciferases use different substrates and thus can be differentiated by their enzymatic specificities. Performing most dual-luciferase assays involves adding two reagents to each sample and measuring luminescence following each addition. Addition of the first reagent activates the firefly luciferase reaction; addition of the second reagent extinguishes firefly luciferase activity and initiates the second luciferase reaction (*Renilla* luciferase). One such assay is the Dual-Luciferase® Reporter Assay System (Cat.# E1910), which measures both luciferase activities sequentially from a single sample. This system requires cell lysis prior to performing the assay and requires the use of reagent injectors with multiwell plates.

The Dual-Glo® Luciferase Assay System also measures both firefly and *Renilla* luciferase activities from a single sample and provides longer luminescence duration (in other words, a longer luciferase signal half-life), which is useful when performing reporter assays in multiwell plates. As with other reagents designed for use in multiwell plates, the Dual-Glo® Assay works directly in mammalian cell culture medium without prior cell lysis.

Another dual-luciferase assay option, the Nano-Glo® Dual-Luciferase® Reporter Assay System (Cat.# N1610), involves firefly and NanoLuc® luciferases. This combination of reporters and assay has several advantages, including lower background levels due to better quenching of firefly luciferase activity and higher sensitivity due to brighter NanoLuc® luminescence. The long signal half-lives (approximately 2 hours for firefly luciferase and NanoLuc® luciferase) make this assay particularly useful for high-throughput screening. In addition, the lower firefly luciferase background and brighter NanoLuc® signal allows greater flexibility in assay configuration. Either NanoLuc® or firefly luciferase can be used as the experimental reporter, with the other luciferase used as the normalization control, or both reporter genes can be used as the experimental reporter, providing you with more information from a single set of assays.

In general, dual-reporter assays improve experimental accuracy and efficiency by: i) reducing variability that can obscure meaningful correlations; ii) normalizing interfering phenomena that may be inherent in the experimental system; and iii) normalizing differences in transfection efficiencies between samples. In addition, dual-reporter assays can reduce the number of nonrelevant hits (i.e., "false" hits) due to direct interaction of compounds with the reporter protein in high-throughput screenings. The use of co-incidence reporters—reporters that have dissimilar profiles of compound interference—can help differentiate compounds that modulate the biological pathway of interest from those that affect the stability or activity of the reporter enzyme.

Reducing Variability

Because cells are complex micro-environments, significant variability can occur between samples within an experiment and between experiments performed at different times. Challenges include maintaining uniform cell density and viability between samples and accomplishing reproducible transfection of exogenous DNA. The use of multiwell plates introduce variables such as edge effects, which are brought about by differences in heat distribution and humidity across a plate. Dual-reporter assays can control for much of this variability, leading to more accurate and meaningful comparisons between samples (Hawkins *et al.* 2002; Hannah *et al.* 1998; Wood, 1998; Faridi *et al.* 2003).

Dual-Color Assays

In some cases, researchers may prefer to activate both luciferase assays simultaneously by adding a single reagent. This reduces total assay volume and liquid-handling requirements. Promega scientists have developed click beetle luciferases, which are related to firefly luciferase and can be differentiated by the color of luminescence: red and green. The sequences and structures of these Chroma-Luc™ luciferases are nearly identical, with only a few amino acid substitutions necessary to create the different colors. This structural similarity means that both the control and experimental reporters are likely to respond similarly to biochemical changes within the cell, resulting in more accurate normalization to the control reporter. For situations where genetic recombination between the two luciferase genes is a concern, Promega scientists have developed two genes encoding the green-emitting reporter: one that is nearly identical to the luciferase emitting red luminescence, and one that is maximally divergent from it. The Chroma-Luc™ genes encoding both the red- and green-emitting luciferases are codon-optimized for mammalian cells.

The Chroma-Glo™ Luciferase Assay System measures Chroma-Luc™ activities in multiwell plates. The Chroma-Glo™ Reagent formulation supports optimal reaction kinetics for both reporters simultaneously and works directly in culture medium. Because color differentiation is required for the Chroma-Glo™ Assay, a luminometer capable of using colored optical filters is required. Since the light is transmitted through optical filters, sensitivity relative to other assay methods is reduced. Both red- and green-emitting Chroma-Luc™ luciferase activities are detectable using optical filters when the relative concentrations differ by up to 100-fold. This is less than dual-luciferase assays that use chemical differentiation, where the relative concentrations may differ by more than 1,000-fold.

Additional Resources for Dual-Reporter and Dual-Color Assays

Technical Bulletins and Manuals

TM040	*Dual-Luciferase® Reporter Assay System Technical Manual*
TM426	*Nano-Glo® Dual-Luciferase® Reporter Assay System Technical Manual*
TM058	*Dual-Glo® Luciferase Assay System Technical Manual*
TM062	*Chroma-Glo™ Luciferase Assay System Technical Manual*
TM259	*pGL4 Luciferase Reporter Vectors Technical Manual*

Online Tools

Dual-Luciferase® Reporter Assay System video
A more powerful dual luciferase assay chalk talk.
Nano-Glo® Dual-Luciferase® Reporter Assay System manual protocol video.
Nano-Glo® Dual-Luciferase® Reporter Assay System injectors protocol video.

Citations

Guo, R. *et al.* (2013) Novel microRNA reporter uncovers repression of Let-7 by GSK-3β. *PLos ONE* **8**, e66330.

Members of the let-7 microRNA family are thought to act as tumor suppressors. The authors of this paper describe a sensitive luciferase-based reporter assay for detecting let-7 miRNA activity in cells. The reporter construct was based on the pmirGLO Vector, which contains firefly luciferase as the reporter gene and *Renilla* luciferase as an internal control. The authors inserted let-7 miRNA target sites at the 3′ end of the firefly luciferase gene. Interaction of let-7 miRNA with these target sequences resulted in reduced luciferase activity. The authors used the reporter construct to screen a kinase inhibitor library for compounds that repressed let-7 activity in ovarian cancer cells and identified GSK-3β as a potential target for therapeutics.

PubMed Number: 23840442

Mateo, M. *et al.* (2011) VP24 is a molecular determinant of Ebola virus virulence in guinea pigs. *J. Infect. Dis.* **204**, 1011–20.

The authors used the Dual-Glo® Luciferase Assay System to measure a pISG54 promoter-driven firefly luciferase gene and pRL-TK plasmid constitutively expressing *Renilla* luciferase, and either a plasmid expressing the corresponding variants of Ebola virus (EBOV) structural protein VP24 construct (phCMV-EBOV-VP24) or empty phCMV in HEK 293T and GPC-16 transfected cells. Cells

were stimulated with interferon 24 hours post-transfection, harvested 16 hours later and assayed for dual-luciferase activity using the Dual-Glo® Luciferase Assay System. Data indicated that mutations in the V24 protein were associated with EBOV virulence but that this virulence was not linked to the interferon-antagonist function of V24 protein.

PubMed Number: 21987737

Elbashir, S.M. *et al.* (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494–8.

In this landmark paper decribing RNA interference in mammalian cells, firefly and *Renilla* luciferase gene products were targeted for degradation. NIH/3T3, HEK293, HeLa S3, COS-7 and S2 cells were transfected with 1µg of pGL2-Control or pGL3-Control Vector, 0.1µg pRL-TK Vector and 0.21µg siRNA duplex targeting either firefly or *Renilla* luciferase. The Dual-Luciferase® Reporter Assay System was used 20 hours post-transfection to monitor luciferase expression. Transfection with 21bp dsRNA caused specific degradation of a targeted sequence. This was the first demonstration of the RNAi effect in mammalian cells.

PubMed Number: 11373684

Promega Publications

NIH Chemical Genomics Center: Small-molecule screening for investigating fundamental biological questions.

Deciphering the pGL4 Vector code.

pGL4 Vectors: A new generation of luciferase reporter vectors.

Increased *Renilla* luciferase sensitivity in the Dual-Luciferase® Reporter Assay System.

Introducing Chroma-Luc™ technology.

Dual-Glo™ Luciferase Assay System: Convenient dual reporter measurements in 96- and 384-well plates.

C. Live-Cell Substrates

Researchers strive to monitor cellular activities with as little impact on the cell as possible. The endpoint of an experiment, however, sometimes requires complete disruption of cells so that the environment surrounding the reporter enzyme can be carefully controlled. Recently, Promega scientists developed a variety of live-cell substrates to monitor *Renilla* and firefly luciferase activity without disrupting cells.

Renilla luciferase requires only oxygen and coelenterazine to generate luminescence, providing a simple luciferase system to measure luminescence from living cells. Unfortunately, coelenterazine is unstable in aqueous solutions, so it can be difficult and inconvenient to measure *Renilla* luciferase. EnduRen™ and ViviRen™ Live Cell Substrates overcome this difficulty and easily generate luminescence from live cells expressing *Renilla* luciferase. Because luminescence is generated from living cells, these substrates are ideal for multiplexing with assays that determine viable cell number.

An alternative to live-cell substrates is a secreted form of reporter protein, which can be quantified by measuring reporter activity in the cell culture medium. NanoLuc® luciferase is ideally suited for both standard (lytic) and secretion-based (nonlytic) reporter gene applications. Promega scientists have fused NanoLuc® luciferase to an N-terminal secretion signal, resulting in a secreted form (secNluc) that does not require cell lysis prior to or during the reporter assay.

Additional Resources for Live-Cell Substrates

Technical Bulletins and Manuals

TM244 *EnduRen™ Live Cell Substrate Technical Manual*

TM064 *ViviRen™ Live Cell Substrate Technical Manual*

Citations

Dragulescu-Andrasi, A. *et al.* (2011) Bioluminescence resonance energy transfer (BRET) imaging of protein-protein interactions within deep tissues of living subjects. *Proc. Natl. Acad. Sci. USA* **108**, 12060–5.

The authors constructed red light-emitting reporter systems based on bioluminescence resonance energy transfer (BRET) for ratiometric imaging of protein:protein interactions in cell culture and deep tissues of small animals. These BRET systems consist of *Renilla* luciferase (RLuc) variants as BRET donors, combined with two red fluorescent proteins as BRET acceptors. They used the EnduRen™ Live Cell Substrate to produce a red-shift emission maxima optimal for deep-tissue imaging. To demonstrate this capability, the authors imaged HT-1080 cells accumulating in the lungs of nude mice. The cells expressed BRET fusion proteins in the context of rapamycin-induced association of FK506-binding protein 12 (FKBP12) and FKBP12 rapamycin-binding domain. Mice were injected with luciferase substrate at 1.5 hours after cell injection to produce a bioluminescence image. Their data suggest that the BRET systems could be used for drug screening and target validation applications.

PubMed Number: 21730157

Promega Publications

In vivo evaluation of regulatory sequences by analysis of luciferase expression.

Measuring *Renilla* luminescence in living cells.

Perform multiplexed cell-based assays on automated platforms.

Bioluminescence imaging of live trout for virus detection using EnduRen™ Live Cell Substrate.

D. Bioluminescent Reporters to Normalize for Changes in Cell Physiology

Events associated with cell physiology can affect reporter gene expression. Of particular concern is the effect of cytotoxicity, which can mimic genetic downregulation when using a single-reporter assay. Reporter assays that can be multiplexed with a cell viability or cytotoxicity assay

allow independent monitoring of both reporter expression and cell viability to avoid data misinterpretation (Farfan *et al.* 2004). The use of multiplexed assays allows correlation of events within cells, such as the coupling of target suppression by RNAi, to the consequences on cellular physiology (Hirose *et al.* 2002).

Luminescent cell viability or cytotoxicity assays, such as the CellTiter-Glo® Luminescent Cell Viability Assay and CytoTox-Glo™ Cytotoxicity Assay, use a stabilized firefly luciferase to generate a luminescent signal that is indicative of cell health. Because these assays contain firefly luciferase, they cannot be directly combined with a firefly luciferase reporter assay. However, the assays can be readily combined with nondestructive luciferase assays. For example, expression of *Renilla* luciferase may be quantitated by adding EnduRen™ or ViviRen™ Live Cell Substrate to the culture medium and measuring luminescence. When the reporter measurements are completed, the CellTiter-Glo® or CytoTox-Glo™ Reagent is added to the sample to inactivate *Renilla* luciferase and initiate luminescence, which is indicative of cell viability. Alternatively, secNluc can used as the reporter, and activity can be measured from an aliquot of culture medium using the Nano-Glo® Luciferase Assay System.

Fluorescent cell viability and cytotoxicity assays are also available to monitor cell health and normalize single-reporter assay results. For example, the CellTiter-Fluor™ Cell Viability Assay is a nonlytic, fluorescence assay that measures the relative number of viable cells in a population. The CellTiter-Fluor™ Substrate enters intact cells, where it is cleaved by a live-cell protease that is restricted to intact cells to generate a fluorescent signal proportional to the number of living cells. The number of nonviable cells does not affect fluorescence because the live-cell protease becomes inactive upon loss of membrane integrity and leakage into the culture medium. Similarly, the CytoTox-Fluor™ Cytotoxicity Assay uses a fluorogenic peptide substrate to measure the relative number of dead cells in cell populations by quantifying a distinct protease activity associated with cytotoxicity. These two assays are combined in the MultiTox-Fluor Multiplex Cytotoxicity Assay. Finally, the CellTox™ Green Cytotoxicity Assay uses a proprietary dye that is excluded from viable cells but preferentially binds to DNA from dead cells. Upon DNA binding, fluorescence of the dye is substantially enhanced, and the resulting fluorescence is proportional to the level of cytotoxicity. These assays are well suited for multiplexing with homogeneous luciferase assay reagents such as Bright-Glo™, Steady-Glo® and ONE-Glo™ Luciferase Assay Systems (Zakowicz *et al.* 2008) as well as the *Renilla*-Glo® Luciferase Assay System.

E. Bioluminescent Reporters to Monitor RNA Interference

RNA interference (RNAi) is a phenomenon by which double-stranded RNA complementary to a target mRNA can specifically inactivate a gene by stimulating degradation of the target mRNA. As such, RNAi has emerged as a powerful tool to analyze gene function. Since its report in *C. elegans* (Fire *et al.* 1998), RNAi has been reported in a variety of organisms, including zebrafish, planaria, hydra, fungi, *Drosophila* and plant and mammalian systems. These phenomena have been collectively termed RNA silencing and appear to use a common set of proteins and short RNAs. These processes are mechanistically similar but not identical.

Bioluminescent reporters have been harnessed to study RNAi. For example, the pmirGLO Dual-Luciferase miRNA Target Expression Vector quantitatively evaluates microRNA (miRNA) activity through insertion of miRNA target sites downstream or 3′ of the firefly luciferase gene. Reduced firefly luciferase expression indicates binding of endogenous or introduced miRNAs to the cloned miRNA target sequence. This vector also encodes *Renilla* luciferase (*hRluc-neo*) as a control reporter for normalization and selection. For more information about the RNAi process and technologies that can be used to design, synthesize and evaluate short interfering RNAs (siRNAs) and small hairpin RNAs (shRNAs), refer to the RNA Interference chapter.

F. Bioluminescent Reporters in Cell-Signaling Assays

Luciferase reporter assays are widely used to investigate cellular signaling pathways and as high-throughput screening tools for drug discovery (Brasier *et al.* 1992, Zhuang *et al.* 2006). Synthetic constructs with cloned regulatory elements directing reporter gene expression can be used to monitor signal transduction and identify the signaling pathways involved. By linking luciferase expression to specific response elements (REs) within the reporter construct, transfecting cells with this construct, subjecting the transfected cells to a particular treatment, and then measuring reporter activity, researchers can determine what REs are used, and thus, what signaling pathways are involved. The use of inhibitors and siRNAs can be used to confirm what factors are involved in this response.

To speed this type of research, Promega scientists have designed several convenient pGL4 Vectors with your choice of a number of response elements and regulatory sequences to take advantage of the benefits of the pGL4 Vector backbone and *luc2P* gene. See Table 8.1. Many of these vectors encode the hygromycin-resistance gene to allow selection of stably transfected cell lines. Alternatively, Promega offers cell lines that are already stably transfected with pGL4-based vectors with specific response elements. See the Luciferase Reporter Cell Lines section.

Bioluminescent reporters also enable characterization of nuclear receptors, a class of ligand-regulated transcription factors that sense the presence of steroids and other molecules inside the cell. Nuclear receptors typically reside in the cytoplasm and are often complexed with associated regulatory proteins. Ligand binding triggers translocation into the nucleus, where the receptors bind specific response elements via the DNA-binding domain, leading to upregulation of the adjacent gene. Bioluminescent reporters can be harnessed to identify and characterize nuclear receptor agonists, antagonists, co-repressors and

co-activators using a universal receptor assay, which is similar in many ways to the two-hybrid assay. In a two-hybrid assay, two proteins that are thought to interact are expressed as fusion proteins, one fused with the DNA-binding domain (DBD) of the yeast GAL4 transcription factor and the other fused to the VP16 activation domain. Protein:protein interaction brings the two domains together to yield expression of a reporter gene downstream of tandem GAL4-binding sites and a minimal promoter. The universal nuclear reporter assay can be thought of as a "one-hybrid" assay, where the ligand-binding domain (LBD) of a nuclear receptor replaces the bait and prey proteins and VP16 activation domain (Figure 8.7).

To perform the universal nuclear receptor assay, simply cotransfect the cell line of interest with a construct encoding the LBD-GAL4 DBD fusion protein and a suitable reporter vector with multiple copies of the GAL4 upstream activation sequence (UAS) upstream of the promoter and reporter gene. Two to three days post-transfection, treat cells with the test compounds of interest, then measure luciferase activity. This approach allows you to convert any cell line into a nuclear receptor-responsive cell line, which you can use to identify receptor agonists, antagonists, co-activators and co-repressors. You can even perform mutagenesis on the ligand-binding domain to determine the effect in your responsive cell line without interference from the endogenous receptor. An example of a suitable reporter construct is the pGL4.35[*luc2P*/9X*GAL4*UAS/Hygro] Vector (Cat.# E1370), which contains nine copies of the GAL4 UAS immediately upstream of a minimal promoter driving expression of *luc2P* reporter gene. For added convenience, Promega offers HEK293 cells that are stably transfected with the pGL4.35[*luc2P*/9X*GAL4*UAS/Hygro] Vector: the GloResponse™ 9X*GAL4*UAS-*luc2P* HEK293 cells. For more information, see the Luciferase Reporter Cell Lines section.

Promega offers a number of additional reagents to simplify universal nuclear receptor assays. The pBIND-ERα Vector (Cat.# E1390) contains the yeast Gal4 DBD and an estrogen receptor ligand-binding domain (ER-LBD) gene fusion, and the pBIND-GR Vector (Cat.# E1581) contains the yeast Gal4 DBD and glucocorticoid receptor ligand-binding domain (GR-LBD) gene fusion. Promega also offers the pFN26A (BIND) *hRluc*-neo Flexi® Vector (Cat.# E1380), which allows expression of a fusion protein comprised of the GAL4 DBD, a linker segment and an in-frame protein-coding sequence under the control of the human cytomegalovirus (CMV) immediate early promoter. You simply clone the DNA fragment encoding the ligand-binding domain of your receptor into SgfI and PmeI sites at the 5′ and 3′ ends of a lethal barnase gene, which acts as a positive selection for successful ligation of the insert. Each BIND vector contains a *Renilla* luciferase/neomycin resistance co-reporter for normalization of transfection efficiency or construction of a double-stable cell line without the need for additional cloning.

Bioluminescent reporters also are useful for studying G protein-coupled receptors (GPCRs), which regulate a wide-range of biological functions and are one of the most important target classes for drug discovery (Klabunde *et al.* 2002). The firefly luciferase-based GloSensor™ cAMP Assay provides a sensitive and easy-to-use format to interrogate overexpressed or endogenous GPCRs that signal via changes in intracellular cAMP concentration. The assay uses genetically encoded biosensor variants comprised of cAMP-binding domains fused to mutant forms of *Photinus pyralis* luciferase. cAMP binding induces conformational changes that promote large increases in light output. Following pre-equilibration with the GloSensor cAMP Reagent, cells transiently or stably expressing the biosensor variant can be used to assay GPCR function using a nonlytic assay format, enabling kinetic measurements of cAMP accumulation or turnover in living cells. Moreover, the assay offers a broad dynamic range, with up to 500-fold changes in light output. Extreme sensitivity allows detection of G_i-coupled receptor activation or inverse agonist activity in the absence of artificial stimulation by compounds such as forskolin. For more information, visit the GloSensor™ Technology page.

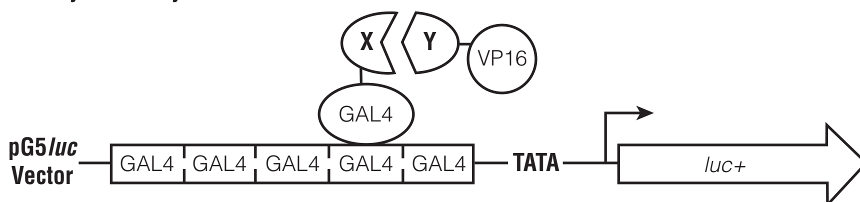
Similar luminescent biosensors exist to detect site-specific protease activity. To detect the protease, an oligonucleotide encoding the protease recognition sequence is cloned into the pGloSensor™-10F Linear Vector (Cat.# G9461), and the GloSensor™ protein containing the protease site of interest is synthesized in a cell-free protein expression system and subsequently used as a protease substrate. Cleavage of the protease recognition sequence leads to activation of the GloSensor™ protein and light emission. The level of luminescence correlates to protease activity. Luminescent biosensors to detect cGMP and caspase-3/7 activity in vivo are also available.

Additional Resources for Bioluminescent Reporters in Cell-Signaling Assays

Technical Bulletins and Manuals

9PIE847	*pGL4.29[luc2P/CRE/Hygro] Vector Product Information*
9PIE848	*pGL4.30[luc2P/NFAT-RE/Hygro] Vector Product Information*
9PIC935	*pGL4.31[luc2P/GAL4UAS/Hygro] Vector Product Information*
9PIE849	*pGL4.32[luc2P/NF-κB-RE/Hygro] Vector Product Information*
9PIE134	*pGL4.33[luc2P/SRE/Hygro] Vector Product Information*
9PIE135	*pGL4.34[luc2P/SRF-RE/Hygro] Vector Product Information*
9PIE137	*pGL4.35[luc2P/9XGAL4UAS/Hygro] Vector Product Information*
9PIE136	*pGL4.36[luc2P/MMTV/Hygro] Vector Product Information*

A. Two-Hybrid Assay



B. Universal Nuclear Receptor Assay

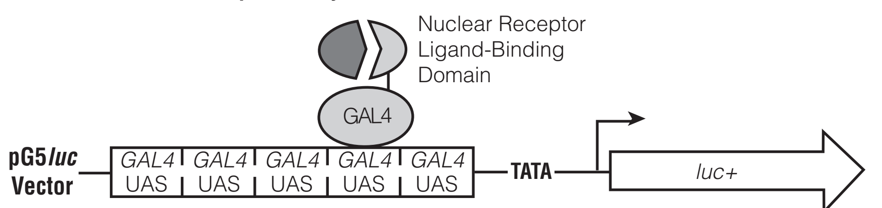


Figure 8.7. The two-hybrid assay and universal nuclear receptor assay. Panel A. The traditional two-hybrid assay. The pG5*luc* Vector contains five GAL4 upstream activator sequences (UAS) upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene. Interaction between the two test proteins, expressed as GAL4-X and VP16-Y fusion proteins, results in an increase in luciferase transcription and expression. **Panel B.** The universal nuclear receptor assay. The ligand-binding domain of the nuclear receptor replaces the bait and prey proteins and VP16 activation domain. Within the cell, binding of the appropriate ligand to the nuclear receptor-GAL4 fusion protein releases any co-repressors bound to the ligand-binding domain. Co-activators help recruit the transcription machinery to the luciferase reporter gene, resulting in luciferase expression and an increase in luminescence.

Promega Publications

Novel pGL4 reporter vector panel for profiling cellular stress and chemical toxicity.

G. Luciferase Reporter Cell Lines

The GloResponse™ Cell Lines contain optimized luciferase reporter technology integrated into a cell line. These cells use the destabilized and optimized *luc2P* gene for greater sensitivity and shorter induction times compared to native reporter enzymes. The GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line, NFκB-RE-*luc2P* HEK293 Cell Line and CRE-*luc2P* HEK293 Cell Line allow rapid and convenient analysis of cell signaling through the NFAT, NF-κB or cAMP response pathways, respectively, via activation of a luciferase reporter gene. Non-native activators of these pathways, including GPCRs, can be studied after the appropriate proteins are introduced by transfection.

GPCR signaling pathways can be categorized into three classes based on the G protein α-subunit involved: G_s, $G_{i/o}$ and G_q. The GloResponse™ CRE-*luc2P* HEK293 Cell Line can be used to study and configure screening assays for G_s- and $G_{i/o}$-coupled GPCRs, which signal through cAMP and the cAMP reponse element (CRE). For G_q-coupled GPCRs, which signal through calcium ions and NFAT-RE, use the GloResponse™ NFAT-RE-*luc2P* HEK293 Cell Line. GPCR assays that use the GloResponse™ Cell Lines are amenable to high-throughput screening. These assays typically have greater response dynamics (fold of induction) than other assay formats and generate high-quality data as indicated by the high Z′ values.

The GloResponse™ Cell Lines were generated by clonal selection of HEK293 cells stably transfected with pGL4-based vectors carrying specific response elements for the pathway of interest. These cell lines incorporate improvements developed for the pGL4 Vectors for enhanced performance and reduced background reporter expression. The destabilized *luc2P* luciferase reporter improves responsiveness to transcriptional dynamics and is codon-optimized for enhanced expression in mammalian cells. The result is cell lines with very high reporter induction levels when the pathway of interest is activated.

Additional Resources for Luciferase Reporter Cell Lines

Technical Bulletins and Manuals

TB362	*GloResponse™ CRE-luc2P HEK293 Cell Line Technical Bulletin*
TB363	*GloResponse™ NFAT-RE-luc2P HEK293 Cell Line Technical Bulletin*
TB380	*GloResponse™ NFκB-RE-luc2P HEK293 Cell Line Technical Bulletin*

H. Bioluminescent Reporters to Study Protein Dynamics

The rate of protein turnover is tightly regulated for many signaling proteins. Protein stabilization and subsequent accumulation occur in response to changing cellular conditions, resulting in activation of downstream transcriptional events. Promega offers reporter vectors to study the rate of protein turnover of two key signaling proteins involved in response to cellular stress: hypoxia-inducible factor-1A (HIF1A) and nuclear factor erythroid 2-related factor-2 (NRF2). The pNLF1-HIF1a

[CMV/neo] Vector (Cat.# N1381) enables simple quantification of intracellular HIF1A protein levels to study the dynamics of this signaling protein in mediating cellular response to hypoxia. The vector encodes NanoLuc® luciferase fused to the C-terminus of the HIF1A protein under control of the CMV promoter. By using a constitutive promoter such as CMV to drive expression of the HIF1A-NanoLuc® fusion protein, changes in light output correlate to dynamic changes in protein levels. Similarly, the pNLF1-NRF2 [CMV/neo] Vector (Cat.# N1391) enables simple quantification of intracellular NRF2 protein levels to study the dynamics of this signaling protein in mediating cellular response to oxidative stress. The vector encodes NanoLuc® luciferase fused to the C-terminus of the NRF2 protein under the control of the CMV promoter and is supplied with the pKEAP1 [CMV/Hygro] Vector for proper regulation of intracellular NRF2 levels.

Additional Resources for Bioluminescent Reporters to Study Protein Dynamics

Technical Bulletins and Manuals

9PIN138 *pNLF1-HIF1a [CMV/neo] Vector Product Information*

9PIN139 *pNLF1-NRF2 [CMV/neo] Vector Product Information*

Promega Publications

Detection of HIF1α expression at physiological levels using NanoLuc® Luciferase and the GloMax® Discover System

Measuring intracellular protein lifetime dynamics using NanoLuc® luciferase.

I. Promega Reporter Vectors and Assays

There are many factors to consider when choosing a reporter vector and assay. The tables in this section show the various features of reporter vectors, including the reporter gene, presence of a multiple cloning region, gene promoter, protein degradation sequences and mammalian selectable marker (Tables 8.1, 8.2 and 8.3), as well as the features of Promega reporter assays (Table 8.4). These tables will help you choose a reporter vector or assay. For the most up-to-date selection of reporter vectors and assays, visit the Promega web site. For a step-by-step guide to help you choose the best luciferase reporter vector for your studies, use the Luciferase Reporter Vector Selector.

IV. Getting the Most Out of Your Genetic Reporter Assays

A. Introduction

When performed properly, experiments using genetic reporters can yield tremendous amounts of information. However, there are several important considerations when designing and performing these types of experiments to ensure that the data are sound.

B. Choice of Reporter Gene and Assay

The ideal reporter gene is one that is not endogenously expressed in the cell of interest and is amenable to assays that are sensitive, quantitative, rapid, easy, accurate, reproducible and safe. One of the first considerations when performing genetic reporter assays is choosing a reporter gene. The characteristics and advantages of common luminescent reporters are discussed earlier; see the Luciferase Genes and Vectors section.

Also important when choosing a reporter methodology is deciding if a single reporter is sufficient or if the greater information density of a dual assay is preferred. The use of two reporters can improve experimental outcomes by two means: 1) normalizing interfering phenomena that may be inherent in biological systems such as cytotoxicity; and 2) reducing random variability due to factors such as differences in cell density and viability, differences in transfection efficiency and variability due to "edge effects" brought about through differences in heat capacity and humidity across a multiwell plate. For more information, see the Luciferase Reporter Assays and Protocols section.

Another important consideration is reporter assay characteristics such as signal half-life, signal intensity, lytic or nonlytic protocol, and compatibility with multiwell plates in a high-throughput format. Assays based on a single reporter provide the quickest and least expensive means to acquire data. However, because cells are inherently complex, the quantity of data available from a single reporter may be insufficient to achieve reliable results. A discussion of the most common reporter assays is provided in the Luciferase Genes and Vectors section.

C. Reporter Construct Design

A common question when designing reporter vectors for promoter dissection is “What sequences should I clone into my vector?”. Unfortunately, there is no one correct answer. The necessary sequences depend on the biological question you are trying to answer and the vector into which you are cloning. An advantage of transgenic reporter assays is that you can control which elements are examined. You might include the entire proximal promoter (including ~1kb upstream of the promoter), a specific promoter subsection or as little as a single response element. When generating a transcriptional fusion or using a vector with no transcription start site, you might include the +1 transcription start site. The reporter might include the 5′ UTR if you want to understand how this sequence may affect promoter activity, but keep in mind that UTR sequences also can affect post-transcriptional regulation. The reporter might include an intron and all or part of one or both flanking exons to study RNA splicing or characterize regulatory elements contained within the intron (but if the exons include coding sequence, be sure to clone the reporter gene in frame). The reporter might contain the 3′ UTR to focus on post-transcriptional regulation through miRNA effects. You might clone any combination of sequences from your gene of interest to look at the integration of regulatory pathways; reporter assays allow this flexibility and refined experimental design.

D. Controls

The proper controls are an important part of reporter assays. The most important is a control using untransfected cells to define the background signal in the assay (from luminometer noise or reagent background). Generally, background luminescence is inconsequential, and the signal:background ratio is quite high in luciferase assays. Additional controls may include the parent vector used to prepare the reporter vector (minus any inserts) and a positive control vector. Measuring luciferase activity from the parent vector allows you to discount any reporter response due to the vector backbone, not the insert; experimental results can be expressed as the ratio of experimental vector response to parent vector response. The same function is generally provided by the second reporter in a dual-reporter assay when using matched vectors (i.e., pGL4 firefly and *Renilla* vectors).

A reporter vector with a relatively strong promoter can serve as a positive control for luciferase expression and detection in the cell line of interest and can be used to normalize for interfering phenomena. However, strong promoters, such as cytomegalovirus (CMV) and SV40 promoters, can easily interfere with transcriptional activity of weaker promoters by sequestering transcription factors and are more likely to be regulated by experimental treatments due to the high number of transcription factor-binding sites. In general, we recommend avoiding promoters with the highest activities in your cells. Vectors with weaker promoters often are a better choice, and even vectors without a promoter yield sufficient luciferase activity for normalization purposes in most cells and are less likely to be regulated by the treatment.

E. Transfection Parameters

Transfection is necessary to introduce the reporter vector into a cell. Transient transfection is the most common method, but stable transfection should be considered if you are performing the same reporter assay frequently. Both approaches have advantages and disadvantages. Transient transfection allows you to vary the reporter vectors and vector ratios, but cells must be transfected for each set of experiments and will lose the reporter vector over time. Transfection efficiency can be low for primary cells and some cell lines and can vary considerably. Often a second reporter is cotransfected into cells to normalize for differences in transfection efficiency. These differences can be assessed relatively in cell lysates by comparing reporter activity or assessed absolutely by estimating the percentage of cells expressing the transferred gene by in situ staining. A second reporter also can help to determine if a response was due to cell toxicity and not a promoter-specific event. Because transfection can be stressful, cells are often allowed to recover prior to experimental treatment. Stable transfection eliminates variability in transfection efficiency and the stress of transfection but requires additional time and effort to select stably transfected cells.

The optimal transfection reagent and conditions depend on the cell line used and must be determined empirically. Prior to performing an experiment, we recommend using a control reporter vector with a strong promoter in the cell line of interest and varying the transfection parameters, such as vector ratio, amount of DNA and amount of transfection reagent, to determine the optimal transfection conditions. Efficient transfection can be critical when using less sensitive reporters such as chloramphenicol acetyltransferase (CAT) or β-galactosidase but is less of a concern with sensitive reporters such as luciferase. For a detailed discussion of transfection, see the Transfection chapter of the Protocols and Applications Guide.

Two important factors when transfecting cells prior to a dual-reporter assay are the reporter vector ratio and relative promoter strengths in the cell line of interest. The optimal ratio often is related to promoter strength and must be determined empirically. It is important, especially if you must use a reporter vector with a strong promoter, to transfect cells with several different ratios of reporter vectors. For assays using pGL4 Vectors with firefly and *Renilla* luciferases, we recommend a 20:1 ratio as a good starting point, but the ratio could be as high as 1,000:1, especially if the promoter of one vector is dramatically stronger than that of the other. The ratio also might be higher if NanoLuc® luciferase is used as one of the reporter proteins due to the enzyme's bright signal. For vectors with promoters of equal strengths, the ratio might be closer to 1:1. When performing vector titrations, be sure to transfect all cells with a constant amount of DNA to minimize differences in transfection efficiency due to differing DNA amounts. The ideal ratio will provide moderate but consistent detection of the experimental luciferase signal that is not influenced by the amount of control luciferase vector present. Regardless of the vector ratio, be sure to use enough control plasmid to provide a signal that is below the saturation point of the luminometer and at least three standard deviations above background levels.

F. Assay Timing

The times between plating and transfection, transfection and experimental treatment, and treatment and reporter assay need to be consistent within a set of experiments to minimize variability and improve assay precision and accuracy. When plating cells prior to transfection, take into account the growth rate of the cells so that cells reach proper confluency at the time of transfection. If necessary, allow time for cells to recover after transfection and the reporter to reach steady state levels of expression. During initial assay optimization, perform a time course to determine the time of peak reporter expression. The optimal time between treatment and reporter assay depends on a number of factors, including the kinetics of your system, longevity of the change you are monitoring (i.e., the assay window) and stability of the reporter protein.

For early-responding genes, we recommend a reporter with a short protein half-life such as the Rapid Response™ luciferase genes, which encode protein degradation

sequences (PEST; Li *et al.* 1998; or CL1; Gilon *et al.* 1998) to destabilize the reporter protein. The Rapid Response™ luciferase genes (*luc2P* and *luc2CP*) respond more quickly and with greater magnitude to changes in transcriptional activity than their more stable counterpart (*luc2*). A destabilized version of NanoLuc® luciferase (*NlucP*) is also available in certain pNL Vectors (see Table 8.2). The onset of the response is more tightly coupled to the induction event. The use of more stable luciferase versions will result in a later and longer response with a wider assay window, so assay timing is not as critical. In these cases, reporter assays are commonly performed 12–24 hours after treatment.

G. Cells and Cell Culture Conditions

Cells and cell culture conditions used to dissect a promoter can affect assay design and results. Three types of cells are commonly used: 1) fibroblasts, which are easy to maintain and amenable to most reporter assays but may not express necessary co-factors or be as biologically relevant as other cells; 2) cancer cells, which may be more relevant and are easy to use; and 3) primary cells, which may be the most biologically relevant but often are difficult to obtain, maintain and transfect. Cell cultures should not be confluent during the experiment, since confluent cells can exhibit differences in metabolism, gene expression and physiological response compared to preconfluent cell cultures. Likewise, cells at higher passage numbers may not behave in the same way as cells at lower passage numbers. If necessary, grow and freeze a large quantity of cells at a lower passage number to ensure that your experiments are performed with cells at a similar passage number. Cells should be healthy and, ideally, easy-to-transfect. When repeating an experiment, be sure to replicate cell culture and transfection conditions as closely as possible to ensure consistency. Be aware that components of cell culture media can affect the intensity and duration of the luminescent signal of a luciferase assay. For example, phenol red can decrease relative luminescence.

H. Special Considerations for High-Throughput Assays

Due to their sensitivity and broad linearity, luminescent reporter genes are particularly well suited to a wide range of high-throughput applications, including genetic reporter assays and luciferase-based assays to measure diverse targets such as kinases, cytochrome p450s, proteases, apoptosis, cell viability and cytotoxicity. We recommend using the optimized luciferase genes described earlier in this chapter—*luc2*, *hRluc* and NanoLuc® luciferase—for high-throughput screening (HTS) due to the substantial increase in expression efficiency. The resulting increase in assay sensitivity is particularly important in HTS, where the trend is toward miniaturization to reduce consumption of costly screening compounds by using high-density multiwell plates (e.g., 1,536- or 3,456-well format). NanoLuc® luciferase has the added benefits of higher specific activity for a brighter signal and enhanced thermal stability for fewer false hits in primary screens.

The destabilized versions of these luciferases (*luc2P*, *luc2CP*, *hRlucP*, *hRlucCP* and *NlucP*) enhance reporter response dynamics, reduce assay time and minimize secondary effects that may arise from the prolonged incubation of cells with screening compounds.

The reporter gene assay is also an important consideration for HTS. Many newer luciferase assays were developed with HTS applications in mind. They often have a longer signal half-life (≥2 hours) so that the assay reagent can be added to all wells of a set of plates and then luminescence measured without a noticeable drop in luminescence intensity from beginning to end. The longer signal half-life eliminates the need for a luminometer with reagent injector. These newer assays often are more tolerant of inhibitors and nonoptimal reaction conditions. For this reason, the reagent can be added directly to cells in growth medium, and there is no need to remove the medium, wash cells or perform a separate cell lysis step. This higher tolerance for inhibitors also reduces the risk of false screening hits due to luciferase inhibition by compound libraries used in contemporary drug discovery to identify agonists and antagonists of pharmacological targets.

The risk of false screening hits often can be decreased further by multiplexing your reporter gene assays with cell viability or cytotoxicity assays to control for artifacts that may arise due to changes in cell health and by using co-incidence reporters that have dissimilar profiles of compound interference to help differentiate compounds that modulate the biological pathway of interest from those that affect the stability or activity of the reporter protein. An example of a co-incidence reporter system involves stoichiometric expression of firefly luciferase and PEST-destabilized NanoLuc® luciferase from the same promoter using ribosome skipping mediated by the P2A peptide. Luciferase activities are measured sequentially from the same sample using the Nano-Glo® Dual-Luciferase® Reporter Assay System, a homogeneous lytic assay performed in an "add-read-add-read" format. For more information, view the Reducing False Hits in HTS Using a Coincidence Reporter System presentation.

To improve data quality and maximize the amount of data obtained from a single sample during high-throughput screenings, researchers often multiplex assays. Multiplexing requires that the assay chemistries are compatible and that the separate assay signals can be distinguished using different detection modalities such as fluorescence and luminescence. For noncompatible assays, measurements must be done sequentially or a portion of the sample must be physically removed to a separate plate. One easy way to separate a sample is to use a secreted reporter, which accumulates in the culture medium and can be measured in a nondestructive, nonlytic way by using an aliquot of culture medium to perform the assay. NanoLuc® luciferase is readily available in a secreted form.

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VI. Vector Tables

Table 8.1. pGL4 Luciferase Reporter Vectors.

Vector	Reporter Gene	Multiple Cloning Region	Protein Degradation Sequence	Gene Promoter	Mammalian Selectable Marker
pGL4.10	*luc2*	Yes	No	No	No
pGL4.11	*luc2P*	Yes	hPEST	No	No
pGL4.12	*luc2CP*	Yes	CL1-hPEST	No	No
pGL4.13	*luc2*	No	No	SV40	No
pGL4.14	*luc2*	Yes	No	No	Hygro
pGL4.15	*luc2P*	Yes	hPEST	No	Hygro
pGL4.16	*luc2CP*	Yes	CL1-hPEST	No	Hygro
pGL4.17	*luc2*	Yes	No	No	Neo
pGL4.18	*luc2P*	Yes	hPEST	No	Neo
pGL4.19	*luc2CP*	Yes	CL1-hPEST	No	Neo
pGL4.20	*luc2*	Yes	No	No	Puro
pGL4.21	*luc2P*	Yes	hPEST	No	Puro
pGL4.22	*luc2CP*	Yes	CL1-hPEST	No	Puro
pGL4.23	*luc2*	Yes	No	minP	No
pGL4.24	*luc2P*	Yes	hPEST	minP	No
pGL4.25	*luc2CP*	Yes	CL1-hPEST	minP	No
pGL4.26	*luc2*	Yes	No	minP	Hygro
pGL4.27	*luc2P*	Yes	hPEST	minP	Hygro
pGL4.28	*luc2CP*	Yes	CL1-hPEST	minP	Hygro
pGL4.29	*luc2P*	No	hPEST	minP + CRE	Hygro
pGL4.30	*luc2P*	No	hPEST	minP + NFAT RE	Hygro
pGL4.31	*luc2P*	No	hPEST	adenovirus major late + GAL4 UAS	Hygro
pGL4.32	*luc2P*	No	hPEST	minP + NF-kB RE	Hygro
pGL4.33	*luc2P*	No	hPEST	serum response element	Hygro
pGL4.34	*luc2P*	No	hPEST	SRF RE	Hygro
pGL4.35	*luc2P*	No	hPEST	*GAL4* UAS	Hygro
pGL4.36	*luc2P*	No	hPEST	murine mammary tumor virus long terminal repeat	Hygro
pGL4.37	*luc2P*	No	hPEST	minP + antioxidant RE	Hygro
pGL4.38	*luc2P*	No	hPEST	minP + p53 RE	Hygro
pGL4.39	*luc2P*	No	hPEST	minP + ATF6 RE	Hygro
pGL4.40	*luc2P*	No	hPEST	minP + metal RE	Hygro
pGL4.41	*luc2P*	No	hPEST	minP + heat shock RE	Hygro
pGL4.42	*luc2P*	No	hPEST	minP + hypoxia RE	Hygro

Vector	Reporter Gene	Multiple Cloning Region	Protein Degradation Sequence	Gene Promoter	Mammalian Selectable Marker
pGL4.43	*luc2P*	No	hPEST	minP + xenobiotic RE	Hygro
pGL4.44	*luc2P*	No	hPEST	minP + AP1 RE	Hygro
pGL4.45	*luc2P*	No	hPEST	minP + interferon stimulated RE	Hygro
pGL4.47	*luc2P*	No	hPEST	minP + Sis-inducible element RE	Hygro
pGL4.48	*luc2P*	No	hPEST	minP + SMAD3/SMAD4 binding element RE	Hygro
pGL4.49	*luc2P*	No	hPEST	minP + TCF-LEF RE	Hygro
pGL4.50	*luc2*	No	No	CMV	Hygro
pGL4.51	*luc2*	No	No	CMV	Neo
pGL4.52	*luc2P*	No	hPEST	minP + STAT5 RE	Hygro
pGL4.53	*luc2*	No	No	phosphoglycerate kinase (PGK)	No
pGL4.54	*luc2*	No	No	thymidine kinase (TK)	No
pGL4.70	*hRluc*	Yes	No	No	No
pGL4.71	*hRlucP*	Yes	hPEST	No	No
pGL4.72	*hRlucCP*	Yes	CL1-hPEST	No	No
pGL4.73	*hRluc*	No	No	SV40	No
pGL4.74	*hRluc*	No	No	HSV-TK	No
pGL4.75	*hRluc*	No	No	CMV	No
pGL4.76	*hRluc*	Yes	No	No	Hygro
pGL4.77	*hRlucP*	Yes	hPEST	No	Hygro
pGL4.78	*hRlucCP*	Yes	No	No	Hygro
pGL4.79	*hRluc*	Yes	No	No	Neo
pGL4.80	*hRlucP*	Yes	hPEST	No	Neo
pGL4.81	*hRlucCP*	Yes	CL1-hPEST	No	Neo
pGL4.82	*hRluc*	Yes	No	No	Puro
pGL4.83	*hRlucP*	Yes	hPEST	No	Puro
pGL4.84	*hRlucCP*	Yes	CL1-hPEST	No	Puro

Table 8.2. pNL Reporter Vectors.

Vector	Reporter Gene	Multiple Cloning Region	Protein Degradation Sequence	Gene Promoter	Mammalian Selectable Marker
pNL1.1*Nluc*]	*Nluc*	Yes	No	No	No
pNL1.1.CMV[*Nluc*/CMV]	*Nluc*	No	No	CMV	No
pNL1.2[*NlucP*]	*NlucP*	Yes	hPEST	No	No
pNL1.3*secNluc*]	*secNluc*	Yes	No	No	No
pNL1.3.CMV [*secNluc*/CMV]	*secNluc*	No	No	CMV	No
pNL2.1[*Nluc*/Hygro]	*Nluc*	Yes	No	No	Hygro
pNL2.2[*NlucP*/Hygro]	*NlucP*	Yes	hPEST	No	Hygro
pNL2.3[*secNluc*/Hygro]	*secNluc*	Yes	No	No	Hygro
pNL3.1[*Nluc*/minP]	*Nluc*	Yes	No	minP	No
pNL3.2.CMV	*NlucP*	No	hPEST	CMV	No
pNL3.2.NF-κB-RE [*NlucP*/NF-κB-RE/ Hygro]	*NlucP*	No	hPEST	minP	Hygro
pNL3.2[*NlucP*/minP]	*NlucP*	Yes	hPEST	minP	No
pNL3.3[*secNluc*/minP]	*secNluc*	Yes	No	minP	No
pNLCoI1[*luc2*-P2A-*NlucP*/Hyg]	*luc2*, *NlucP*	Yes	Yes for *NlucP*, No for *luc2*	No	Hygro
pNLCoI2[*luc2*-P2A-*Nluc*/minP/Hyg]	*luc2*, *NlucP*	Yes	Yes for *NlucP*, No for *luc2*	minP	Hygro
pNLCoI3[*luc2*-P2A-*NlucP*/CMV/Hyg]	*luc2*, *NlucP*	No	Yes for *NlucP*, No for *luc2*	CMV	Hygro
pNLCoI4[*luc2*-P2A-*NlucP*/PGK/Hyg]	*luc2*, *NlucP*	No	Yes for *NlucP*, No for *luc2*	PGK	Hygro

Table 8.3. Other Luciferase Reporter Vectors.

Vector	Reporter Gene	Multiple Cloning Region	Protein Degradation Sequence	Gene Promoter	Mammalian Selectable Marker
pGL3-Basic	*luc+*	Yes	No	No	No
pGL3-Control	*luc+*	Yes	No	SV40	No
pGL3-Enhancer	*luc+*	Yes	No	No	No
pGL3-Promoter	*luc+*	Yes	No	SV40	No
pCBR-Basic	*CBRluc*	Yes	No	No	No
pCBR-Control	*CBRluc*	No	No	No	No
pCBG68-Basic	*CBG68luc*	Yes	No	No	No
pCBG68-Control	*CBG68luc*	No	No	No	No
pCBG99-Basic	*CBG99luc*	Yes	No	No	No
pCBG99-Control	*CBG99luc*	No	No	No	No

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Table 8.4. Luciferase Reporter Assays.

Assay System	Gene Assayed	Single-Sample or Plate Assay	Signal Stability	Live-Cell Assay
Single Reporter				
Luciferase Assay System	*luc, luc+, luc2*	Single or Plate[1]	Short (<0.5h)	No
Steady-Glo® Luciferase Assay System	*luc, luc+, luc2*	Plate[2]	Long (>0.5h)	No
Bright-Glo™ Luciferase Assay System	*luc, luc+, luc2*	Plate[2]	Long (>0.5h)	No
ONE-Glo™ Luciferase Assay System	*luc, luc+, luc2*	Plate[2]	Long (≥45 minutes)	No
ONE-Glo™ EX Luciferase Assay System	*luc, luc+, luc2*	Plate[2]	Long (≥2 hours)	No
Renilla Luciferase Assay System	*Rluc, hRluc*	Single or Plate[1]	Short (<0.5h)	No
Renilla-Glo® Luciferase Assay System	*Rluc, hRluc*	Plate[2]	Long (≥60 minutes)	No
Nano-Glo® Luciferase Assay System	*Nluc, secNluc*	Single or Plate[2]	Long (≥2.0h)	Yes[3]
Dual Reporter				
Dual-Glo® Luciferase Assay System	*luc+, luc2, Rluc, hRluc*	Plate[2]	Long (>0.5h)	No
Dual-Luciferase® Reporter Assay System	*luc+, luc2, Rluc, hRluc*	Single (Cat.# E1910)	Short (<0.5h)	No
		Plate[1] (Cat.# E1980)	Short (<0.5h)	No
Nano-Glo® Dual-Luciferase® Reporter Assay System	*Nluc, luc, luc+, luc2*	Plate[2]	Long (≥2h)	No
Chroma-Glo™ Luciferase Assay System	*CBRluc, CBG99luc, CBG68luc*	Plate[2]	Long (>0.5h)	No
Live-Cell				
EnduRen™ Live Cell Substrate	*Rluc, hRluc*	Plate[1]	Long (>0.5h)	Yes
ViviRen™ Live Cell Substrate	*Rluc, hRluc*	Plate	Short (<0.5h)	Yes

[1]Use with plates only when the luminometer has a reagent injector.

[2]We do not recommend the use of this product with automated reagent injectors.

[3]Use for live-cell assays by quantifying the secreted form of NanoLuc® luciferase in culture medium.