

## 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 (88), they are most frequently used as indicators of transcriptional activity in cells (89). Typically, a reporter gene is joined to a promoter sequence in an expression vector that is transfected. Following transfer, the cells are assayed for the presence of the reporter by directly measuring the amount of reporter mRNA, the reporter protein itself or the enzymatic activity of the reporter protein. An ideal reporter gene is not endogenously expressed in the cell type of interest, and is amenable to assays that are sensitive, quantitative, rapid, easy, reproducible and safe.

The most popular systems for monitoring genetic activity in eukaryotic cells include chloramphenicol acetyltransferase (CAT),  $\beta$ -galactosidase, firefly luciferase, growth hormone (GH),  $\beta$ -glucuronidase (GUS), alkaline phosphatase (AP) and, most recently, green fluorescent protein (GFP) and *Renilla* luciferase (90,91)

A control vector can be used to normalize for transfection efficiency or cell lysate recovery between treatments or transfection experiments (92). Typically, the control reporter gene is driven by a strong, constitutive promoter and is co-transfected with experimental vectors. The experimental regulatory sequences are linked to a different reporter gene so that the relative activities of the two reporter gene products can be assayed individually. Control vectors can also be used to optimize transfection methods. Gene transfer efficiency is typically monitored by assaying reporter activity in cell lysates, or by staining the cells *in situ* to estimate the percentage of cells expressing the transferred gene (80).

### Promega's Reporter Systems

Promega currently offers reporter vectors and assay systems for chloramphenicol acetyltransferase (CAT),  $\beta$ -galactosidase, firefly luciferase, and an integrated dual-reporter assay system for the sequential quantitation of firefly and *Renilla* (sea pansy) luciferases.

### Luciferase Reporter Assay System

The luciferase enzyme used most frequently for reporter gene technology is derived from the coding sequence of the *luc* gene cloned from the firefly *Photinus pyralis* (91,93,94). Compared to CAT, the firefly luciferase protein has a shorter half-life in transfected mammalian cells (95,96), making the

luciferase reporter especially suited for transient assays designed to assess inducible and short-lived effects.

The firefly luciferase enzyme catalyzes a reaction using D-luciferin and ATP in the presence of oxygen and  $Mg^{2+}$ , resulting in light emission. The total amount of light measured during a given time interval is proportional to the amount of luciferase reporter activity in the sample. The assay has been improved by including coenzyme A in the reaction, which provides a longer, sustained light reaction with greater sensitivity (97). Light emission is typically quantified over a defined assay period. The extended "glow" reaction of the enhanced luciferase assay allows for accurate measurement of the luminescence reaction when using a luminometer or scintillation counter. The sensitivity of the luciferase assay is in the subattomole range, approximately 30-1,000 times greater compared to the sensitivity of CAT assays (96). An added advantage is that luciferase assay results can be obtained in minutes compared to hours, or even days, for the radioactive CAT assay. The linear range of the firefly luciferase assay extends over an impressive 8 orders of magnitude of firefly luciferase concentration.

### Dual-Luciferase™ Reporter Assay System

Promega's Dual-Luciferase™ Reporter (DLR™) Assay System combines the speed, sensitivity and convenience of two luciferase reporter enzymes into an integrated, single-tube, dual-reporter assay format. The DLR™ Assay is designed to provide rapid, sequential quantitation of firefly luciferase and sea pansy (*Renilla reniformis*) luciferase in cell lysates or cell-free translation systems. Because the firefly and *Renilla* luciferases are of distinct evolutionary origins, they have dissimilar enzyme structures and substrate requirements. These differences make it possible to selectively discriminate between their respective bioluminescent reactions. Thus, the luminescence from the firefly luciferase reaction may be quenched while simultaneously activating the luminescent reaction of *Renilla* luciferase (the control reporter).

*Renilla* luciferase is a 36kDa monomeric protein that utilizes oxygen and coelenterate luciferin (coelenterazine) to generate light emission (98). In the integrated Dual-Luciferase™ Reporter Assay chemistry, the kinetics of the *Renilla* luciferase reaction provide a glow-type luminescent signal that decays slowly over the course of the measurement. Similar to firefly luciferase, the luminescent reaction catalyzed by *Renilla* luciferase provides high sensitivity and a linear range extending over 7 orders of magnitude of *Renilla* luciferase concentration.

**CAT Reporter Assay System**

The CAT gene is derived from transposon 9 of *E. coli* (99). CAT is a trimeric protein comprising three identical subunits of 25kDa (100). The CAT protein is relatively stable in mammalian cells, although the mRNA has a comparatively short half-life, making the CAT reporter especially suited for transient assays designed to assess accumulation of expressed protein (95).

CAT catalyzes the transfer of the acetyl group from acetyl-CoA to the substrate, chloramphenicol. The enzyme reaction can be quantitated by incubating cell lysates with [<sup>14</sup>C]chloramphenicol and following product formation by physical separation with thin layer chromatography (TLC) or organic extraction (101,102).

 **$\beta$ -Galactosidase Reporter Assay System**

The *E. coli lacZ* gene encodes  $\beta$ -galactosidase, a tetrameric enzyme that catalyzes the hydrolysis of  $\beta$ -galactoside sugars such as lactose. The enzymatic activity in cell extracts can be assayed with various specialized substrates that allow quantitation of enzyme activity using a spectrophotometer, a fluorometer or a luminometer. A major strength of this reporter gene is the ability to easily assay *in situ* expression with histochemical staining (see Figure 6.1).

The  $\beta$ -galactosidase reporter gene is frequently used as a control vector for normalizing transfection efficiency when co-transfected with chimeric DNAs linked to other reporter genes (92). One potential limitation of this reporter gene is that certain mammalian cells have endogenous lysosomal  $\beta$ -galactosidase activity. Enzyme assays performed at a higher pH of 7.3-8.0, or with cell extracts pre-heated to 50°C, preferentially favor the *E. coli* enzyme (88, 103). However, because of endogenous

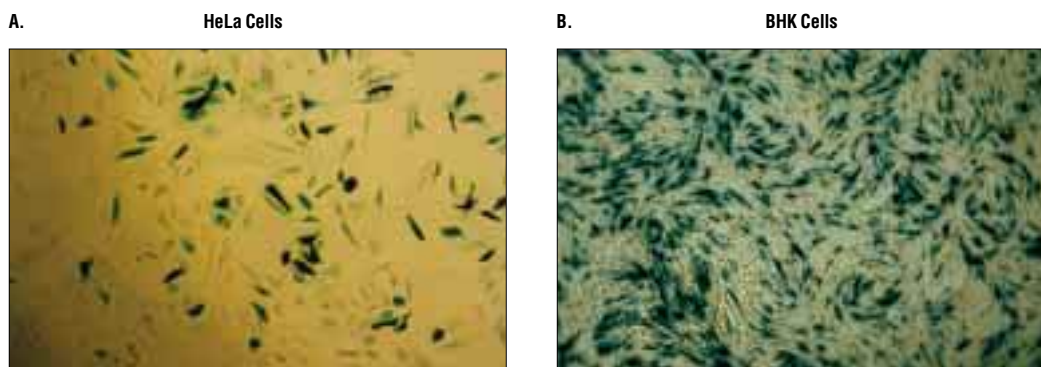
cellular  $\beta$ -galactosidase activity, it is important to include negative control extracts or cells that have not been transfected as comparisons for the cell-free and *in situ* analyses.

**Firefly Luciferase Reporter Gene Systems****Firefly Luciferase Reporter Vectors**

Promega's Luciferase pGL2 and pGL3 Reporter Vectors and Luciferase Assay Reagents provide a basis for rapid, quantitative analysis of factors that potentially regulate gene expression. The pGL2 and pGL3 Luciferase Reporter Vectors contain the cDNA encoding luciferase (*luc*) cloned from the North American firefly (*Photinus pyralis*), as well as numerous features that aid in the characterization and manipulation of cloned regulatory sequences. Changes in luciferase reporter activity directly correlate to the transcriptional activity of the cloned regulatory element when expressed in transfected cells.

All pGL3 Vectors contain a modified firefly luciferase cDNA, designated *luc+*, and a vector backbone that has been designed to provide enhanced reporter gene expression (Figure 6.2). These modifications help to ensure that the luciferase reporter gene, itself, does not contribute spurious transcriptional signals. Further details on these modifications are provided in Technical Manual #TM033.

The combination of modifications embodied in the pGL3 Vector family provides greater flexibility in performing genetic manipulations, minimal background activity and luciferase expression levels that are dramatically higher than previously obtained with the pGL2 Reporter Vectors (104). Using the pGL3 Vectors, it is now possible to obtain



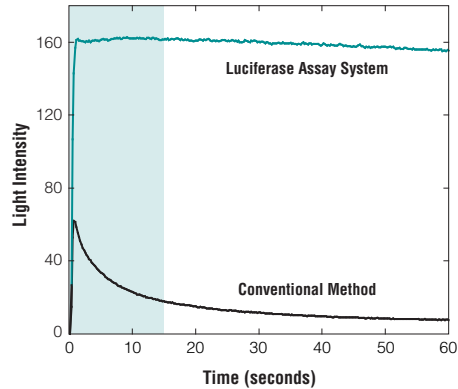
**Figure 6.1. Histochemical staining of HeLa and BHK cells for  $\beta$ -galactosidase activity.** HeLa (Panel A) and BHK (Panel B) cells were plated in 24 well plates and transfected with pCI-*lacZ* vector DNA. BHK cells were transfected with Tfx™-10 Reagent at a 2:1 Reagent:DNA ratio, with 1,000ng of DNA. HeLa cells were transfected with Tfx™-20 Reagent at a 2:1 Reagent:DNA ratio, with 250ng of DNA. The transfections were performed in the absence of serum for one hour. Cells were fixed with glutaraldehyde 48 hours post-transfection and stained for  $\beta$ -galactosidase using standard techniques. The cells expressing  $\beta$ -galactosidase are stained blue.

measurable luciferase expression in cell types that are difficult to transfect, when studying weak promoter elements, or when performing *in vivo* luminescence measurements. It is important to recognize that absolute light unit values and relative expression profiles of reporter vectors will vary between different cell types (104). The appropriate control vector should always be included in experiments utilizing genetic reporter systems.

**General Considerations for Firefly Luciferase Reporter Assays**

Promega's firefly Luciferase Assay System offers several advantages over conventional assays for luciferase (97). The reaction catalyzed by firefly luciferase is oxidation of beetle luciferin with concomitant production of a photon. Under conventional reaction conditions, the oxidation occurs from an enzyme intermediate, luciferyl-AMP. However, recent investigation has revealed that coenzyme A is a substrate in the luminescent reaction. In the presence of CoA, oxidation occurs presumably through luciferyl-CoA. The result is light production without the characteristic self-inhibition of luciferase observed in other assays (97).

The conventional assay for luciferase generates a "flash" of light that rapidly decays after enzyme and substrate are mixed, thus requiring automated injection luminometers for measurements of photon production. Promega's Luciferase Assay System

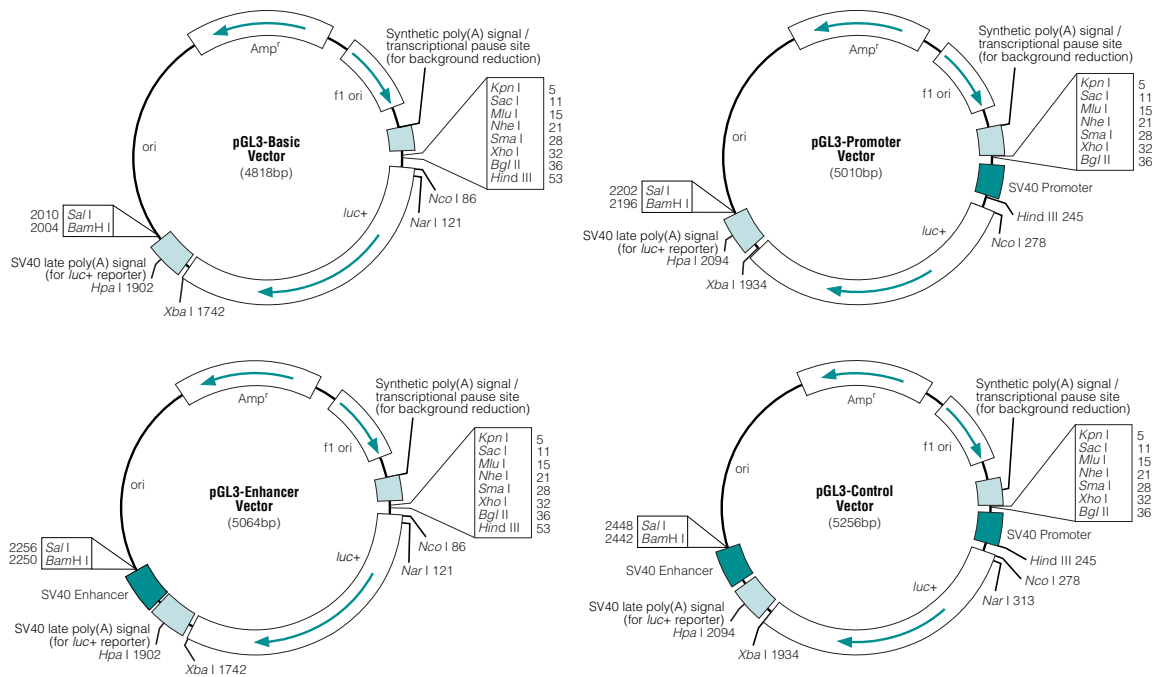


**Figure 6.3. Comparison of Promega's Luciferase Assay System to the conventional luciferase assay method.**

allows for greater enzymatic turnover of luciferase (97), which results in greater light intensity that is nearly constant for measurements of up to several minutes (Figure 6.3).

The constant light intensity generated in Promega's assay eliminates the need for rapid mixing protocols and automated injection devices. The simplified assay procedure is adaptable to different measurement methods for light production, such as scintillation counting or exposure to photographic film.

Please request Promega Technical Bulletins #TB101 or #TB161 for a detailed Luciferase Reporter Assay Protocol.



**Figure 6.2. Circle maps of the pGL3 Vectors.**

## Dual-Luciferase™ Reporter Assay System

### pRL Renilla Luciferase Vectors

The pRL family of *Renilla* luciferase vectors, used with the Dual-Luciferase™ Reporter Assay System, provides options for *Renilla* luciferase expression in transfected mammalian cells. The pRL Vectors may be used in combination with any experimental firefly luciferase vectors to co-transfect mammalian cells. Thus, the expression of *Renilla* luciferase can provide an internal control value to which expression of the experimental firefly luciferase reporter gene may be normalized.

The pRL family of control reporter vectors contain the cDNA encoding *Renilla* luciferase (*Rluc*) cloned from the anthozoan coelenterate *Renilla reniformis*, the sea pansy (105), with some minor modifications for convenience as a genetic reporter. The constitutive expression of *Rluc* is provided by one of several available promoter elements. The pRL Vectors are currently available in three promoter configurations and one promoter-less configuration (Figure 6.4).

### General Considerations for Co-Transfection Experiments

The pRL Vector of choice may be used in combination with any experimental reporter vector to co-transfect mammalian cells. However, it is important to realize the potential for *trans* effects between co-transfection

promoters that may affect reporter gene expression (106). Primarily, this is of concern when working with very strong promoter/enhancer elements resident on one or the other, or both, of the control and experimental reporter vectors. The occurrence and magnitude of such effects will depend on 1) the combination and activities of the genetic regulatory elements present on the co-transfected vectors; 2) the relative ratio of experimental vector to control vector introduced into the cells; and 3) on the cell type itself.

To help ensure independent genetic expression between experimental and control reporter genes, perform preliminary co-transfection experiments to optimize both the amount of vector DNA and the ratio of co-reporter vectors added to the transfection mix. The extreme sensitivity of both the firefly and *Renilla* luciferase assays, and the very large linear range of luminometers (typically 5-6 logs) allows accurate measurement of substantially different experimental and control luminescence values. Therefore, relatively small quantities of a pRL co-reporter vector are needed to provide low-level, constitutive expression of *Renilla* luciferase control activity. Ratios of luciferase co-transfection vectors of 50:1 or greater are feasible, and in some instances will be preferable to aid in suppressing *trans* effects between promoter elements.

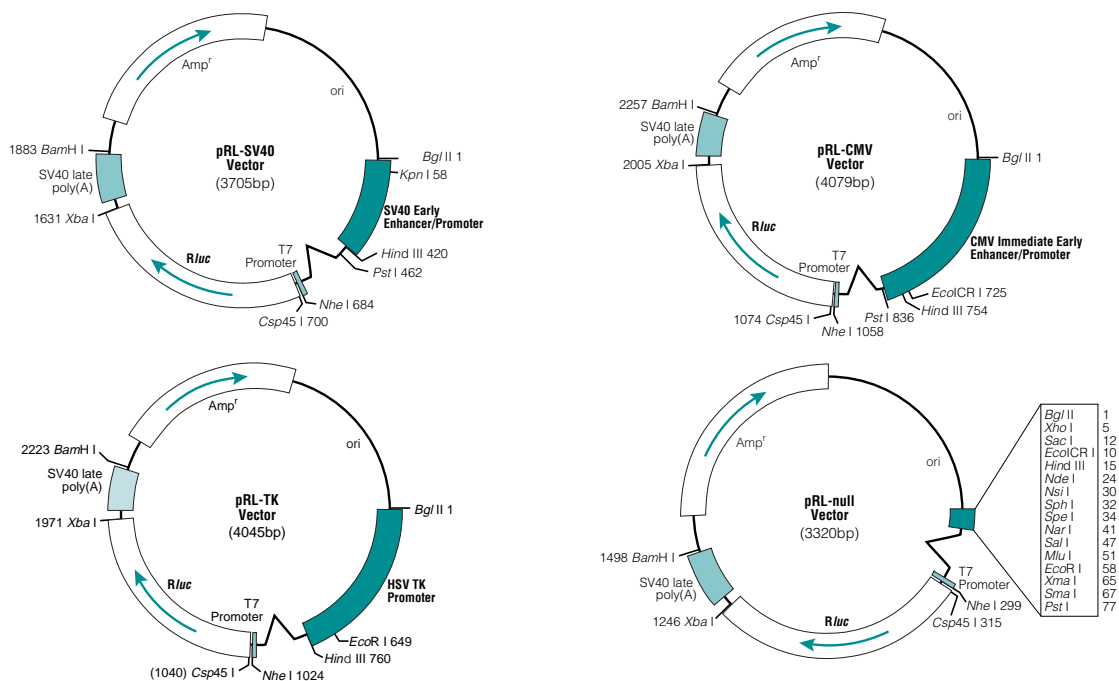
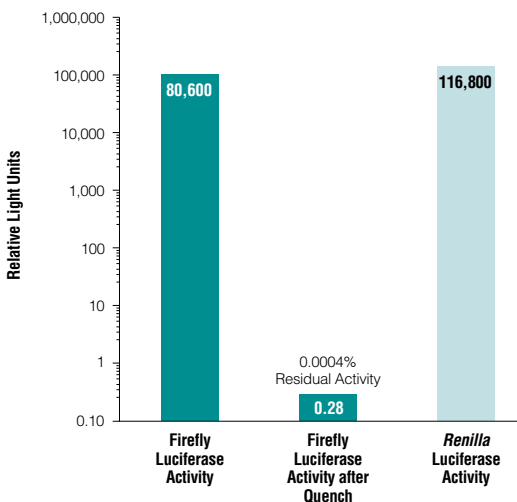


Figure 6.4. Circle maps of the pRL-SV40, pRL-CMV, pRL-TK and pRL-null Vectors.

### General Considerations for the Dual-Luciferase™ Reporter Assay System

The luminescent signal from each of the two luciferase reporter enzymes may be quantitated immediately following lysate preparation without the need for dividing samples or performing additional treatments. The firefly luciferase reporter assay is initiated by adding an aliquot of lysate to Luciferase Assay Reagent II. Quenching of firefly luminescence, and concomitant activation of the *Renilla* luciferase, is accomplished by adding Stop & Glo® Reagent to the sample tube immediately upon completion of the firefly reaction. Luminescence signal from the firefly reaction is quenched by at least a factor of 100,000 (to  $\leq 0.001\%$  residual light output) within one second following the addition of Stop & Glo® Reagent (see Figure 6.5).

Complete activation of *Renilla* luciferase is also achieved within this one-second period. When using a manual luminometer, the time required to quantitate both luciferase reporter activities will be approximately 30 seconds.



**Figure 6.5.** Measurement of luciferase activities before and after the addition of Stop & Glo® Reagent.

### Instrument Considerations

The Turner Designs Model TD-20/20 Luminometer (Promega Cat.# E2061) is ideally suited for low-throughput processing of Dual-Luciferase™ Reporter Assays. The instrument is pre-programmed to complete sequential readings of both firefly and *Renilla* luciferase reporter activities with a single “Start” command. Further, the instrument is programmed to provide the individual and normalized luciferase values, as well as statistical analyses of values measured within replicate groups.

Please request Promega Technical Manuals #TM040 or #TM046 for more information on the Dual-Luciferase™ Reporter Assay System protocol.

## CAT Reporter Gene Systems

### pCAT® Reporter Vectors

Promega provides two families of CAT gene reporter vectors: pCAT® and pCAT® 3 Reporter Vectors. Each family of vectors contains four plasmids which are referred to as pCAT®, or pCAT® 3-Basic (lacking eukaryotic promoter and enhancer sequences), Enhancer (with the SV40 early enhancer element 3' of the CAT gene), Promoter (with the SV40 early promoter driving expression of the CAT gene) and Control (with the SV40 early promoter driving expression of the CAT gene and the SV40 early enhancer 3' of the gene). Figure 6.6 provides vector maps for the pCAT® 3 Vectors; maps for the pCAT® Vectors are provided in Technical Bulletins #TB080-083.

The increase in CAT expression observed with the pCAT® 3 Vectors provides greater sensitivity. It may now be possible to obtain measurable CAT expression in cell types that are difficult to transfect or when studying weak promoter elements. Users of the pCAT® and pCAT®3 Vectors should be aware, however, that relative expression profiles vary between different cell types (107). Therefore, it is important to include the appropriate control vectors in all experiments.

Details on pCAT®3 Reporter Vector cloning strategies and analyses are provided in Technical Manual #TM036.

### CAT Enzyme Assays

Chloramphenicol acetyltransferase (CAT), encoded by a bacterial drug-resistance gene, inactivates chloramphenicol by acetylating the drug at one or both of its two hydroxyl groups (108). This gene is not found in eukaryotes, and therefore eukaryotic cells contain no background CAT activity. This characteristic, along with the sensitivity of the assay for CAT activity, has made the CAT gene the most widely used reporter gene for studies of mammalian gene expression (90,109). CAT activity may be monitored by two alternative methods using Promega's CAT Enzyme Assay System.

Please request Promega Technical Bulletin #TB084 for a detailed CAT assay protocol.

### β-Galactosidase Reporter Gene System

The enzyme β-galactosidase is widely used as a reporter molecule for both *in vitro* and transgenic applications. Promega's pSV-β-Galactosidase Control Vector (Cat.# E1081; Figure 6.7) is designed as a positive control vector for monitoring transfection efficiencies of mammalian cells. The SV40 early promoter and enhancer drive transcription of the bacterial *lacZ* gene, which encodes β-galactosidase. β-Galactosidase is an excellent reporter enzyme (89,110) that can be assayed quickly and directly in cell extracts using a spectrophotometric assay (111), or in fixed cells by *in situ* staining (29).

A protocol for histochemical staining for β-Galactosidase can be found in Promega Technical Bulletin #TB097.

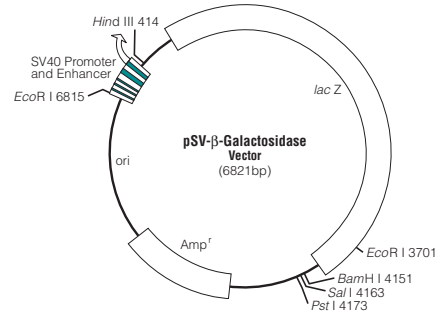


Figure 6.7. Circle map of the pSV-β-Galactosidase Control Vector.

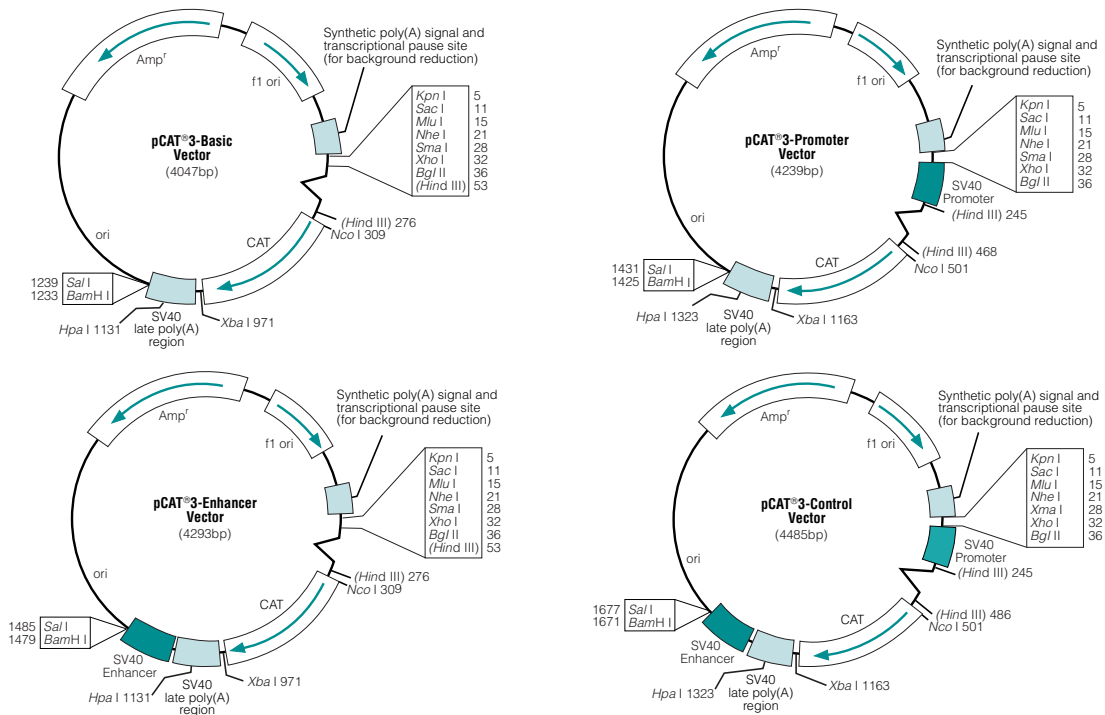


Figure 6.6. Circle maps of the pCAT®3 Vectors.

### Mammalian Expression Vectors

The heterologous expression of proteins in mammalian cells has become an essential technique to study the physiological function of a protein or the effect of post-translational modifications. Promega offers several vectors that can be used for expressing proteins in mammalian cells. Promega's mammalian expression vectors contain the highly active simian virus 40 (SV40) or cytomegalovirus (CMV) promoter and enhancer elements.

The pSI, pCI and pCI-neo Mammalian Expression Vectors, and the pTARGET™ Mammalian Expression Vector System are designed to promote constitutive expression of cloned DNA inserts in mammalian cells (Figure 6.8). Inclusion of the neomycin phos-

photransferase marker in the pCI-neo and pTARGET™ Vectors allows selection of stably transfected mammalian cells in the presence of the antibiotic G-418. We have improved vector design features, such as intron and polyadenylation regions, which provide enhanced RNA stability and subsequent translation. To aid cDNA subcloning, restriction sites in the multiple cloning regions are compatible with the Universal RiboClone® cDNA Synthesis System.

For more information on Promega's Mammalian Expression Vectors, please request Technical Bulletins #TB206 (pSI and pCI Vectors), #TB215 (pCI-neo Vector) or Technical Manual #TM044 (pTARGET™ Vector).

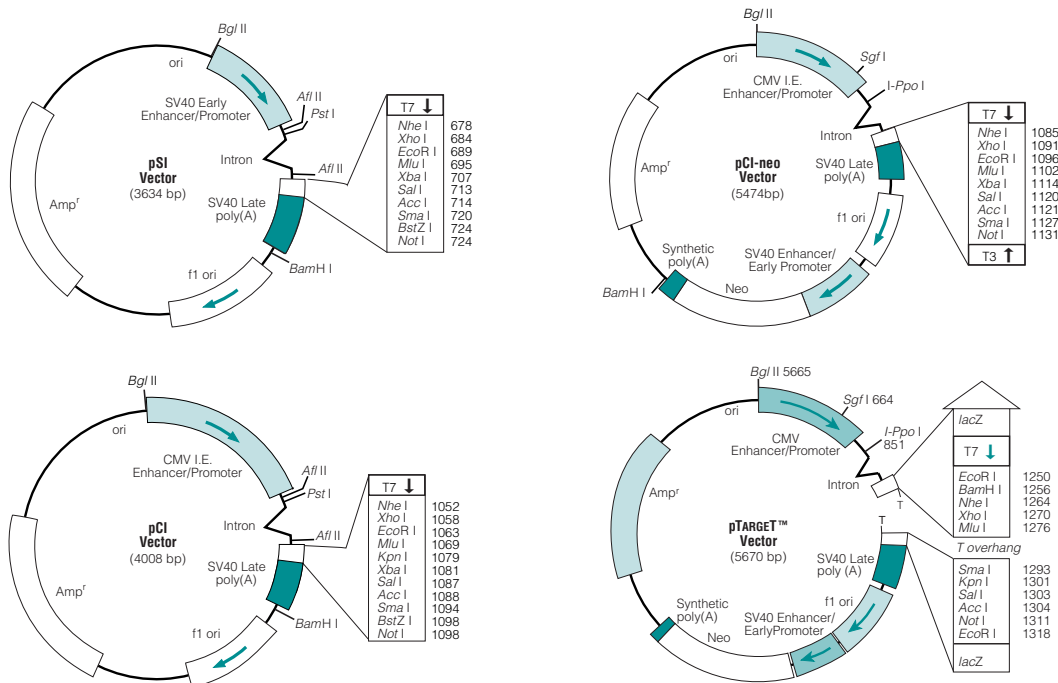


Figure 6.8. Circle maps of the pSI, pCI, pCI-neo and pTARGET™ Vectors.

