Ready-To-Glow™ Secreted Luciferase Reporter System User Manual

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I. Introduction

The **Ready-To-Glow™ Secreted Luciferase Reporter System** is a versatile tool for the systematic analysis of eukaryotic promoters and enhancers. The Secreted Luciferase System uses a secreted luciferase as a reporter molecule to monitor the activity of promoters and enhancers. Such promoter sequences can be cloned into the pMetLuc-Reporter Vector, which is included in the Secreted Luciferase System and is also available separately. The luciferase substrate enables researchers to monitor expression of the pMetLuc-Reporter gene using simple, sensitive, nonradioactive assays of secreted luciferase activity (Figure 1). The Ready-To-Glow Secreted Luciferase System is based on secreted *Metridia* luciferase. It combines the advantage of a live cell assay with the sensitivity of an enzyme-based system—all in a "one-step"–reaction by detecting the activity of the secreted reporter enzyme in the supernatant of transfected cells without the need for cell lysis.

The *Metridia longa* secreted luciferase gene, cloned from the marine copepod *Metridia longa,* encodes a 24 kDa protein containing a N-terminal signal peptide of 17 amino acid residues for secretion (1). This secreted luciferase gene has been sequence-optimized by deleting possible cis-acting sites (splice sites), increasing the overall GC content to prolong mRNA half-life. It has also been human-codon-optimized.

The secreted nature of this luciferase provides several advantages when using this enzyme as a transcription reporter:

- Preparation of cell lysates is not required for analysis.
- The kinetics of gene expression can be studied simply by repeated collection of the culture medium from the same cultures.
- Transfected cells are not disturbed by measurement of luciferase activity in the medium, so a single set of cultures can be used both for the secreted luciferase assay and for further investigations such as DNA/RNA, protein, or cellular analysis.
- Sample collection from the culture medium can be automated by growing cultures and performing the assays in multi-well plates.
- The assay exhibits higher sensitivity compared to fluorescence-based reporter assays.
- Suitable for high-throughput screening applications.

I. Introduction *continued*

1. **Clone response element/ promoter of interest into pMetLuc-Reporter vector**

2. **Transfect host cell line**

3. **At desired time points, assay luciferase activity**
   - Transfer media sample to 96-well plate
   - Add substrate reaction buffer
   - Assay luciferase activity in a luminometer

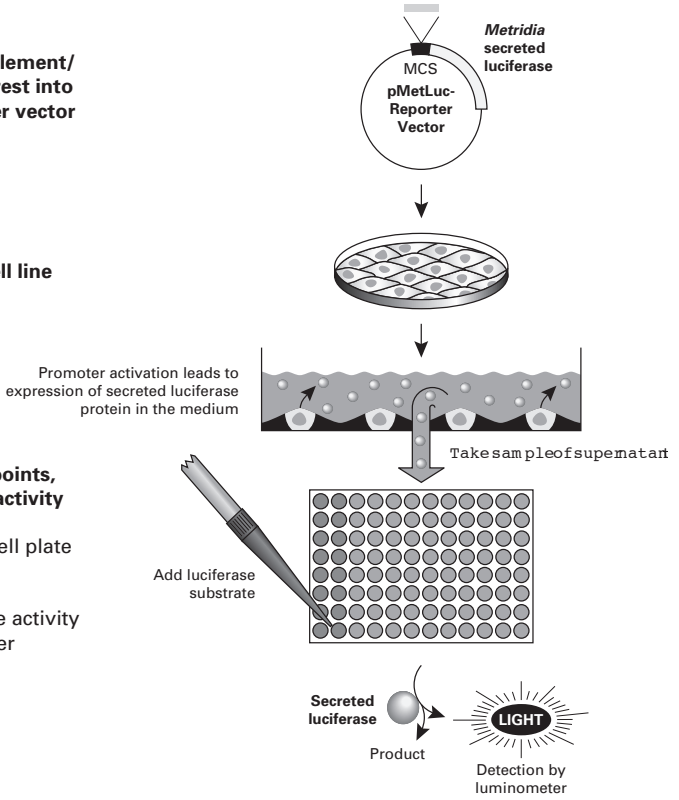

Figure 1. Flowchart of the Ready-To-Glow Secreted Luciferase Reporter Assay procedure.

I. Introduction *continued*

Ready-To-Glow Secreted Luciferase Reporter Vectors

The Ready-To-Glow™ Reporter System includes one reporter and control vector along with sufficient reagents to perform a minimum of 100 reactions. For vector maps and multiple cloning site (MCS) sequences, see Appendix A or visit our web site at **www.clontech.com**.

- **pMetLuc-Reporter** lacks eukaryotic promoter and enhancer sequences, and it contains an MCS that allows promoter DNA fragments to be inserted upstream of the luciferase gene. Enhancers/promoters can be cloned into the MCS upstream of the luciferase gene activity.
- **pMetLuc-Control** is designed to function as a positive control vector. It contains the *Metridia* secreted luciferase gene downstream of the constitutive immeditate early promoter of the cytomegalovirus (P_{CMV}). Both vectors contain SV40 polyadenylation signals downstream of the MetLuc gene which direct proper processing of the 3' end of the MetLuc mRNA. The vector backbone contains an SV40 origin of replication in mammalian cells expressing the SV40 T antigen, a pUC origin of replication for propagation in *E. coli*, and an f1 origin for single-stranded DNA production. A neomycin-resistance cassette (Neo^r) allows stably transfected eukaryotic cells to be selected using G418. This cassette consists of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5, and polyadenylation signals from the Herpes simplex virus thymidine kinase (HSV TK) gene. A bacterial promoter upstream of the cassette expresses kanamycin resistance in *E. coli*.

Some of the key advantages of using our Ready-To-Glow Secreted Luciferase System are highlighted by critical experiments. Our experiments conclude that compared to other non-secreted Luciferase reporters such as *Renilla* luciferase and firefly luciferase, *Metridia* secreted luciferase exhibits a higher signal stability after addition of substrate without compromising signal intensity in the presence of 10% FCS in the media supernatant (Figure 2), allowing easy handling of multiple samples at the same time.

To test the performance of the secreted *Metridia* luciferase gene as a promoter response element reporter, the NFκB response element was cloned into the MCS (multiple cloning site) of the pMetLuc-Reporter Vector and transfected into HeLa cells. Six hr after induction of transiently transfected cells with TNF-α, activation of the NFκB response element was detected by assaying the cell supernatant for *Metridia* secreted luciferase activity. Although signal intensity after substrate addition decreased with time, the overall fold induction was the same even 30 minutes of addition after substrate (Figure 3).

I. Introduction *continued*

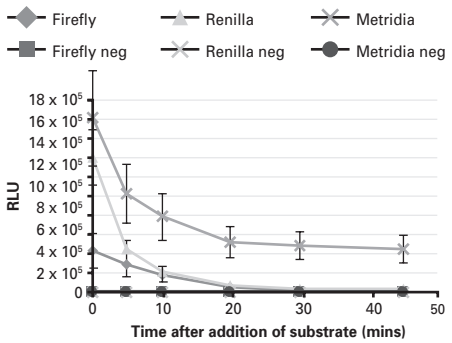

Figure 2. High signal intensity and stability of secreted *Metridia* luciferase. CHO cells were plated into 96-well plates and transiently transfected with CMV-driven constructs encoding non-secreted firefly luciferase, non-secreted *Renilla* luciferase and sequence-optimized secreted *Metridia* luciferase. 24 hr after transfection, luciferase activity in equivalent amounts of samples were analyzed by addition of the recommended substrate. The signal was measured at different timepoints over a period of 45 min following the addition of substrate. neg=negative control

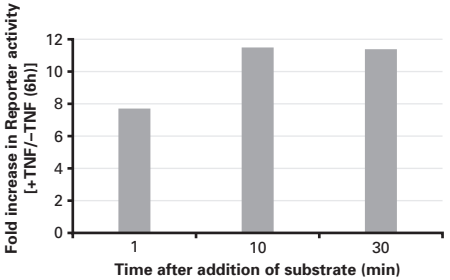

Figure 3. Monitoring promoter activation using the sequence-optimized secreted *Metridia* luciferase reporter. HeLa cells were transiently transfected with a vector construct containing the NFκB response element driving the expression of sequence-optimized secreted *Metridia* luciferase. 24 hr after transfection, the media was removed and replaced by media with or without TNF-α (100 ng/ml) to activate the NFκB signal transduction pathway. Six hr after addition of TNF-α, samples of the media were removed and analyzed for *Metridia* luciferase activity. The fold induction was calculated for different timepoints following the addition of substrate.

I. Introduction *continued*

Ease of use, elimination of cell lysis, signal stability, and high signal intensity make the Ready-To-Glow Secreted Luciferase System a powerful tool for high throughput applications. As shown in Figure 4 (courtesy of Bayer Health Care, Germany), images of a CHO cells in a 1,536 well format, expressing secreted *Metridia* luciferase under the control of a forskolin inducible promoter element, were visualized using a regular, low sensitivity CCD camera. These data confirm that the Ready-To-Glow Secreted Luciferase System is well-suited for high-throughput applications.

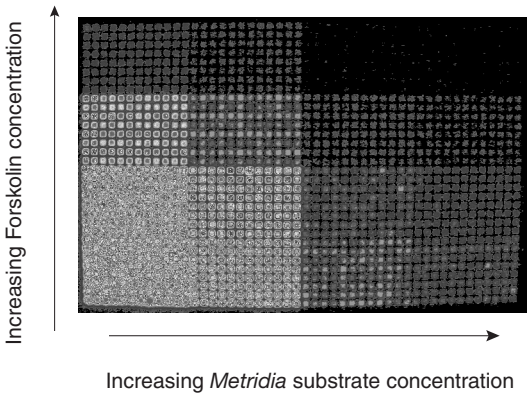

Figure 4. Use of secreted *Metridia* luciferase in a High-Throughput Screening (HTS) application. The figure shows a screen shot from a CCD camera of a 1536-well plate containing stable CHO cells transfected with a forskolin-responsive *Metridia* luciferase gene (pASM-Lu164). 300 cells/well were plated in a 1536 well microtiter plate. Cells were incubated with increasing concentrations of forskolin and the plate was incubated for 4 hr at 37° C. *Metridia* substrate was added in increasing concentrations and the plate was visualized using a CCD camera system (Integration time: 60s). (Courtesy of Bayer Health Care, Germany). This pseudocolor image reflects luminescence intensity. Near white and gray regions are the brightest, while black regions are the least bright.

II. List of Components

The **Ready-to-Glow™ Secreted Luciferase Reporter System** containing the two pMetLuc Vectors and all the components of the Reporter Assay should be stored at –20°C.

The **Ready-to-Glow™ Secreted Luciferase Reporter System** (Cat. Nos. 631730, 631731, & 631732) contains sufficient reagents for 100, 500, and 1,000 assays, respectively.

Cat No. 631730	Cat No. 631731	Cat No. 631732	
10 µg	- -	- -	pMetLuc-Control Vector
10 µg	- -	- -	pMetLuc-Reporter Vector
60 µl	250 µl	500 µl	Substrate Buffer
500 µl	2.5 ml	5 ml	Reaction Buffer
65 µg	275 µg	550 µg	Lyophilized Secreted Luciferase Substrate
100	500	1,000	No. of Reactions

A 10X Substrate Stock Solution combining the Lyophilized Secreted Luciferase Substrate and the Substrate Buffer must be prepared prior to assay time. A detailed protocol for preparing a 10X Substrate Stock Solution is provided in Section IV. C. 2. a.

In addition to the complete system, the two vectors pMetLuc-Control and pMetLuc-Reporter (Cat. No. 631729) and the Ready-To-Glow Reporter Assay (Cat. Nos. 631726, 631727 and 631728) are also available separately.

The **Ready-To-Glow Secreted Luciferase pMetLuc Vector Kit** (Cat. No. 631729) includes:

- 20 µg pMetLuc-Reporter Vector
- 20 µg pMetLuc-Control Vector

The components of the **Ready-To-Glow Reporter Assay** are listed below:

Cat. No. 631726 (100 rxn kit)	Cat. No. 631727 (500 rxn kit)	Cat. No. 631728 (1,000 rxn kit)	
• 60 µl	250 µl	500 µl	Substrate Buffer
• 500 µl	2.5 ml	5 ml	Reaction Buffer
• 65 µg	275 µg	550 µg	Lyophilized Secreted Luciferase Substrate

III. Additional Materials Required

Ready-to-Glow Secreted Luciferase Reporter System assays can be carried out either in 0.5 ml microcentrifuge tubes or multiwell microtiter plates.

Bioluminescence detection of luciferase activity can be performed using either a tube or plate luminometer when reactions carried out in microcentrifuge tubes or in multiwell microtiter plates.

The following type of microtiter plate is recommended:

- **Microlite 1 Luminescence Microtiter 96-well plates, Flat-Bottom** (VWR Scientific Products, Cat. No. 62403-124).
  These opaque white plates, which contain flat-bottom wells, are recommended for bioluminescent assays and detection.

IV. Experimental Design and Sample Preparation

PLEASE READ THROUGH ENTIRE PROTOCOL BEFORE BEGINNING

A. Transfection of Mammalian Cells with pMetLuc Vectors

1. Transfection techniques

   The two pMetLuc Vectors may be transfected into mammalian cells by any standard transfection method. However, when working with a cell line for the first time, we recommend comparing the efficiencies of several transfection protocols using the pMetLuc-Control Vector.

2. Transfection considerations

   a. Perform transfections in triplicate

      Each different construct should be transfected (and subsequently assayed) in triplicate to minimize variability among treatment groups. The primary sources of such variability are differences in transfection efficiencies. If cells are sampled in multiwell plates, we recommend performing transfection in a master stock and then transferring cells from this master stock into the wells of a multiwell plate to avoid variations that may occur if cells in each well were transfected separately.

   b. Normalize for transfection efficiency

      When monitoring the effect of promoter and enhancer sequences on gene expression, it is critical to include an internal control that will distinguish differences in the level of transcription from variability in the efficiency of transfection (Sambrook & Russell, 2001). This is easily done by cotransfecting a second plasmid that constitutively expresses another reporter that can be clearly differentiated from secreted *Metridia* luciferase. The level of this reporter (second reporter) can then be used to normalize the levels of luciferase among different treatment groups. Reporter proteins frequently used for this purpose include *E. coli* β-galactosidase, pCMV-βgal (Cat. No. 631719), and fluorescent proteins, such as AcGFP1 or DsRed-Express (e.g., pAcGFP1-N1, Cat. No. 632469 and pCMV-DsRed, Cat. No. 632416).

B. Proper Use of Controls

1. Negative controls

   A negative control is necessary to measure the background signal associated with the cell culture media. This can be determined by assaying 50 µl of culture medium from cells mock-transfected without vector. The values obtained from such controls should be subtracted from experimental results.

IV. Experimental Design and Sample Preparation *continued*

2. Positive controls
   a. Positive control for transfection and expression of exogenous DNA

      A positive control is necessary to confirm transfection and expression of exogenous DNA and to verify the presence of active secreted *Metridia* luciferase in the culture media. Expression and secretion of functional luciferase in transfected cells can be confirmed by assaying 50 µl of culture medium from cells transfected with the pMetLuc-Control Vector, which contains the luciferase gene under transcriptional control of the CMV promoter. Cells transfected with this plasmid should yield high activity within 24–72 hours (often even earlier) after transfection.

3. Normalizing transfection efficiencies

   It is critical to include an internal control that will distinguish differences in the level of transcription from variability in the efficiency of transfection. See Section A.2.b or Sambrook & Russell (2001) for more information.

C. Protocol and Sample Preparation

1. Transfection and Sampling
   a. Transfect your cells of interest with the construct(s) containing the secreted *Metridia* luciferase reporter gene.
   b. After the time recommended by the transfection protocol for optimal transfection efficiency, remove the medium and either split cells to a multiwell plate, following standard tissue culture protocols or use the cells in the plate right away for the reporter experiment.
   c. Expose the transfected cells to the experimental treatment of your choice based on the studies you would like to perform using the secreted *Metridia* luciferase reporter assay.
   d. Collect 50 µl samples of the media supernatant at different time points after starting the treatment of your choice. These samples can be either assayed for secreted luciferase activity immediately or they can be frozen for subsequent analysis. If multiple samples are collected, they can be transferred into a 96-well plate for further analysis (See Section III for recommended plates). Alternately, they may be frozen for analysis at a later time.

IV. Experimental Design and Sample Preparation *continued*

2. Assay Protocol

a. Preparation of 10X Substrate Stock Solution using Substrate Buffer:

Dissolve the provided Lyophilized Secreted Luciferase Substrate by adding Substrate Buffer [50 µl for 100 reactions, 250 µl for 500 reactions, and 500 µl for 1,000 reactions (Cat. Nos. 631730, 631731 & 631732 respectively)]. DO NOT AGITATE or VORTEX the solution to dissolve the substrate. The substrate is sensitive to oxidation in the presence of air bubbles caused by agitation. Gently mix the substrate in the Substrate Buffer by pipetting. Allowing the substrate in Substrate Buffer to stand for 30 min at room temperature will help dissolve the substrate. The dissolved substrate solution is the 10X Substrate Stock Solution.

b. Preparation of 1X Substrate/Reaction Buffer.

Prepare the 1X Substrate/Reaction Buffer by diluting the 10X Substrate Solution (Step 2.a.) in a ratio of 1:10 in Reaction Buffer. To calculate the total volume of 1X Substrate/ Reaction Buffer needed in your experiment, multiply the number of samples in your experiment by the factor 5. For *eg.,* for a total number of 20 samples, you will have to prepare 100 µl of 1X Substrate/Reaction Buffer by diluting 10 µl of 10X Substrate Stock Solution in 90 µl of Reaction Buffer.

To ensure consistent results, prepare the 1X fresh Substrate/Reaction Buffer every time without introducing air bubbles. This can be achieved by slow pipetting in the Substrate Stock or using the tip to stir the mixture gently instead of using rigorous pipetting. NEVER VORTEX THE SUBSTRATE OR SUBSTRATE/REACTION BUFFER. When the Substrate Stock is mixed with colorless buffer, the resulting 1X solution is light yellow/orange in color. Allow the 1X Substrate/Reaction Buffer to remain at room temperature (RT) for 10 min before use. It can remain at RT for up to 1 hr before use.

c. To test your samples for *Metridia* luciferase activity, we recommend transferring the 50 µl sample into a 96-well plate (See Section III).

d. Add 5 µl of the 1X Substrate/Reaction Buffer (See Step 2.b.) to each sample. (If you have many samples in a 96-well plate, we recommend the use of a multi-channel pipette to reduce the time between addition of substrate and signal detection.)

IV. Experimental Design and Sample Preparation *continued*

3. Bioluminescence Detection Methods
   a. Detection using a plate luminometer
      i. If the assay was not performed in a luminometer-compatible microtiter plate, transfer the entire solution from each well to a suitable plate and place it in the instrument.
      ii. Record light signals with the manufacturer's recommended luminometer settings.

V. Troubleshooting Guide

A. Determining the Linear Range of the Assay

If in doubt about the linear range of the assay, prepare and assay a dilution series using the Positive Control.

B. Little or No Signal From Transfected Cells

1. Ensure that the transfection efficiency has been optimized by using pMetLuc-Control as an internal positive control for luciferase expression.
2. Increase the number and/or density (or concentration) of cells used in transfections.
3. If background signals from positive controls (i.e., cells that are transfected with pMetLuc-Control) are low, incubate the transfected cells in media for a longer time before collecting supernatant samples.
4. Increase the time of transfection before starting the experiment.
5. For detection via a plate luminometer, refer to the instrument instructions for methods to increase the sensitivity of light detection.

C. High Background Signals

The volume of media assayed from experimental cultures may be decreased if the signal is sufficiently high. Alternatively, samples may be diluted.

D. Signal is too High, Exceeding the Linear Range of the Assay

This problem is easily corrected by diluting the sample using the same media used to culture the cells.

E. Recommended Sequencing Primers

We recommend the following primer for sequencing inserts cloned into the pMetLuc-Reporter Vector. (This primer are not available from Clontech.). It may be used to sequence from the 5' region of the *Metridia* Reporter gene region into the MCS.

5'-CACGATGTCGATGTTGGGG-3'
(183–165 in pMetLuc-Reporter Vector)

VI. References

Markova S. V., et al., Cloning and Expression of cDNA for a Luciferase from the Marine copepod *Metridia longa. J. Biol. Chem* **279**(5):3212–3217.

Sambrook, J. & Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory Press (Cold Springs Harbor, NY).

VII. Related Products

For a complete listing of all Clontech products, please visit **www.clontech.com**

Products	Cat. No.
• Ready-To-Glow™ Secreted Luciferase Reporter System	631730 631731 631732
• Ready-To-Glow™ Secreted Luciferase Reporter Assay	631726 631727 631728
• Ready-To-Glow™ Secreted Luciferase Vector Kit	631729
• CalPhos™ Mammalian Transfection Kit	631312
• Clonfectin™	631301
• Human GenomeWalker Kit	638901
• Mouse GenomeWalker Kit	638902
• Rat GenomeWalker Kit	638903
• GenomeWalker Universal Kit	638904

Appendix A: Plasmid Maps & Multiple Cloning Sites

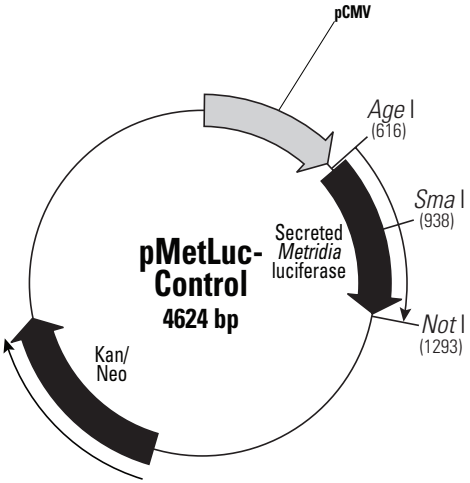

Figure 5. Map of pMetLuc-Control Vector. pMetLuc-Control Vector encodes the codon optimized sequence of the secreted luciferase from the marine copepod *Metridia longa*. This gene encodes a 219 amino acid polypeptide with a molecular weight of 24 kDa. The N-terminus of *Metridia* secreted luciferase contains an N-terminal signal peptide of 17 amino acid residues necessary for secretion. *Metridia longa* secreted luciferase (MetLuc) can be expressed, efficiently secreted, and easily detected in mammalian cell suspensions expressing secreted luciferase.

The complete sequence information for pMetLuc-Control Vector can be downloaded from our web site at **www.clontech.com**.

Appendix A: Plasmid Maps & MCS *continued*

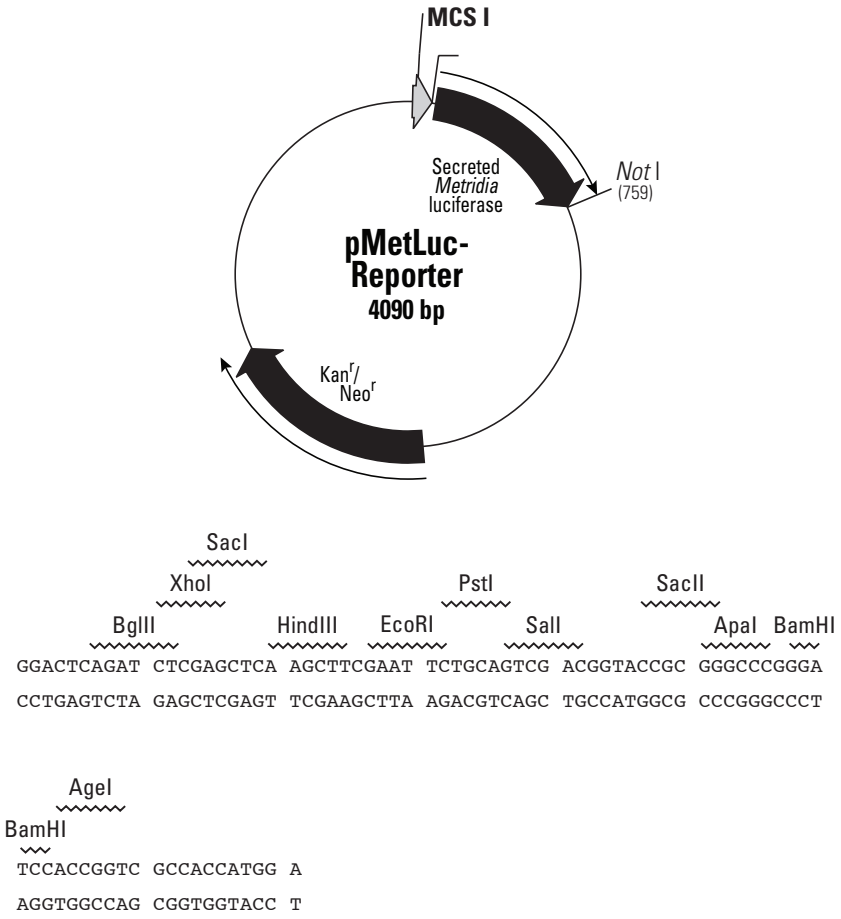

Figure 6. Map of pMetLuc-Reporter Vector. Unique restriction sites are in bold.

The complete sequence information for the pMetLuc-Reporter Vector can be downloaded from our web site at **www.clontech.com**.

Notes