

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



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I. Introduction

A. The Ready-To-Glow Reporter Assay

The **Ready-To-Glow Secreted Luciferase Reporter System** is a versatile tool for the systematic analysis of eukaryotic promoters and enhancers. The system uses secreted *Metridia* luciferase as a reporter molecule to monitor the activity of promoters and enhancers, without the need for cell lysis, by sampling media supernatant. Each Ready-To-Glow system includes a pMetLuc Reporter Vector, which contains either a specific promoter such as NFκB or CRE, or a multiple cloning site where you can clone in the promoter sequence you are interested in. Transfect your cells of interest with the Reporter Vector and culture until you are ready to begin your experiment. Then when you add an inducer, the reporter molecule is expressed and secreted out of the cell into the supernatant. Simply add the luciferase substrate to a sample of the media supernatant, and monitor pMetLuc-Reporter gene expression using a simple, sensitive, non-lysis and non-radioactive assay for secreted luciferase activity without the need for a cell lysis step (Figure 1). You can perform the assay directly in the culture, or to transfer an aliquot of supernatant to another plate to perform the assay. In either case, you will be left with live cells that can be used repeatedly, either for multiple time point assays or for any other analysis on the cells.

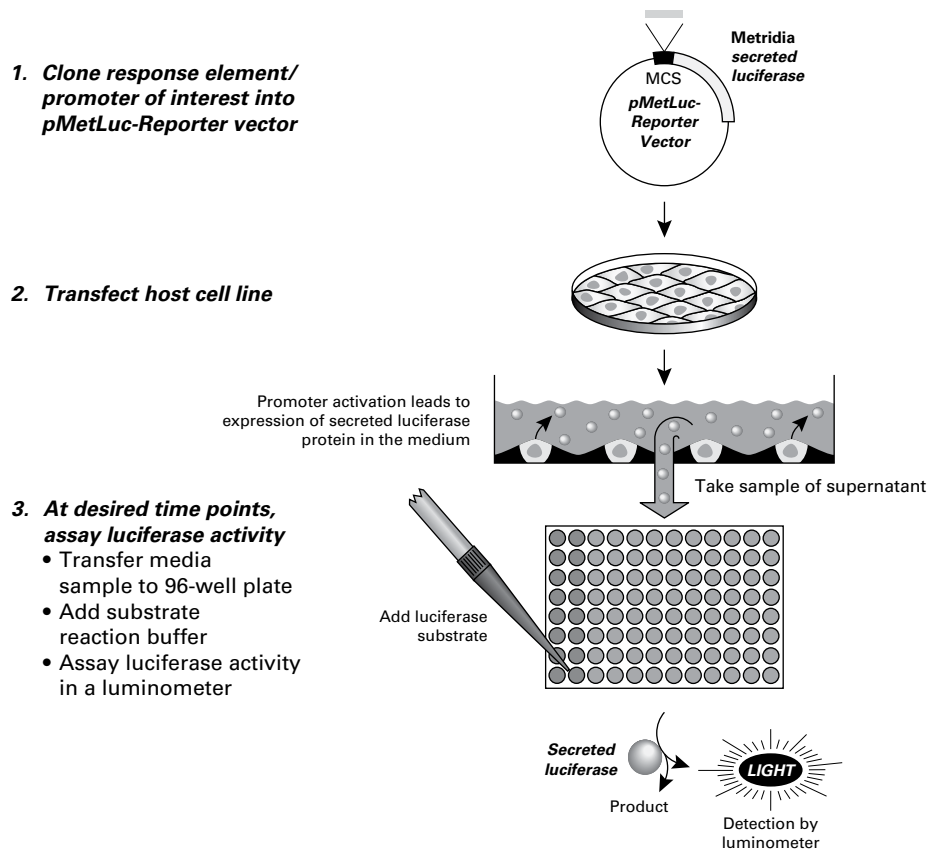


Figure 1. Flowchart of the Ready-To-Glow Secreted Luciferase Reporter Assay procedure. The Ready-To-Glow Secreted Luciferase Reporter System combines the advantages of a live cell assay with the sensitivity of an enzyme-based system—all in a “one-step” reaction which detects the activity of the secreted reporter enzyme in the supernatant of transfected cells without the need for cell lysis. This flowchart describes the process for promoterless pMetLuc-Reporter Vectors. The procedure is identical for promoter-specific pMetLuc-Reporter Vectors, except that Step 1 is eliminated.

I. Introduction continued

The *Metridia longa* secreted luciferase gene was cloned from the marine copepod *Metridia longa*, and encodes a 24 kDa protein containing a 17 amino acid N-terminal signal peptide for secretion (Markova *et al.*, 2004). This secreted luciferase gene has been sequence-optimized by deleting possible *cis*-acting sites (splice sites), and increasing the overall GC content to prolong mRNA half-life. It has also been human codon-optimized to enhance expression in cell lines.

The naturally secreted nature of *Metridia* luciferase provides several advantages for use as a transcription reporter:

- Cell lysis is eliminated.
- Gene expression kinetics studies can be simplified, by repeatedly collecting and assaying media from the same culture (for multiple time points or multiple inducers).
- The same set of cultures can be used both for the secreted luciferase assay *and* for further investigations such as DNA/RNA, protein, or cellular analysis because the transfected cells are not disturbed by measuring luciferase activity in the media.
- Sample (culture media) collection can be automated by growing cultures and performing the assays in multi-well plates.
- The assay is more sensitive than fluorescence-based reporter assays or other cytosolic luciferase reporters.
- Guaranteed low background, because the medium can be removed and replaced at the start of the actual promoter induction experiment.
- A broad dynamic range, because the reporter is secreted. Since there are no proteases in the culture media, every reporter molecule that is expressed during the experiment contributes to the signal.
- The assay is suitable for high-throughput screening applications.

B. The Ready-To-Glow Reporter Vectors

Each Ready-To-Glow Reporter System includes one reporter vector and one control vector, along with sufficient reagents to perform a minimum of 100 reactions.

The reporter vectors are:

- **pMetLuc2-Reporter** contains an MCS that allows promoter or promoter/enhancer elements to be inserted upstream of the secreted *Metridia* luciferase (MetLuc) gene. Activation of the promoter/enhancer elements of interest drives the expression of secreted *Metridia* luciferase, which is detected in the supernatant of the transfected cells.
- **pLVX-MetLuc Vector** contains an MCS that allows promoter or promoter/enhancer elements to be inserted upstream of the secreted *Metridia* luciferase (MetLuc) gene. Activation of the promoter/enhancer elements of interest drives the expression of secreted *Metridia* luciferase, which is detected in the supernatant of the transfected cells. Lentiviral delivery allows you to study your promoter of interest in virtually any cell type, including primary cultures, dividing and nondividing cells, stem cells, terminally differentiated cells, and neuronal cells
- **pNFκB-MetLuc2-Reporter** contains the NFκB promoter element upstream of the secreted *Metridia* luciferase (MetLuc) gene. It is designed to monitor NFκB signal transduction pathways. Adding TNF-α, IL-1, or lymphokine receptor stimulants to the cell culture medium induces the binding of transcription factors to the enhancer element and drives transcription of secreted *Metridia* luciferase.
- **pCRE-MetLuc2-Reporter** contains the CRE promoter element upstream of the secreted *Metridia* luciferase (MetLuc) gene. It is designed to monitor the activation of cAMP binding protein (CREB) and cAMP-mediated signal transduction pathways. Adding cAMP or forskolin to the cell culture medium activates CREB or ATF to bind CRE, and drives transcription of secreted *Metridia* luciferase.

Please visit our website at www.clontech.com/support/vectors.asp for more information and for vector maps.

Each reporter vector comes with a pMetLuc control vector, which is designed to function as a positive control. It contains the secreted *Metridia* luciferase gene downstream of the constitutive immediate early promoter of the cytomegalovirus (pCMV_{IE}), so cells transfected with this construct constitutively express and secrete *Metridia* luciferase.

I. Introduction continued

All of the pMetLuc 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 (pKan^r) upstream of the cassette allows kanamycin resistance in *E. coli*.

The pMetLuc2 vectors also include a synthetic transcription blocker (TB), composed of adjacent polyadenylation and transcription pause sites located upstream of the pCMV_{IE} promoter, which reduces background transcription (Eggermont & Proudfoot, 1993).

C. Characteristics of the Secreted *Metridia* Luciferase Signal

1. High Signal Intensity and Prolonged Stability of the Secreted *Metridia* Luciferase Signal

Compared to non-secreted luciferase reporters such as *Renilla* luciferase and firefly luciferase, secreted *Metridia* luciferase exhibits a higher signal intensity and prolonged stability after substrate addition, making it easy to handle multiple samples at the same time. The signal is stable between 20 and 45 minutes after substrate addition (Figure 2). Furthermore, the presence of 10% FBS in the media supernatant does not compromise signal intensity.

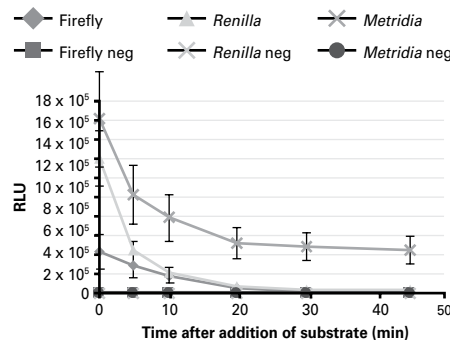


Figure 2. High signal intensity and stability of secreted *Metridia* luciferase. CHO cells were plated into 96-well plates and transiently transfected with CMV-driven constructs encoding non-secreted firefly luciferase, non-secreted *Renilla* luciferase and sequence-optimized secreted *Metridia* luciferase.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

In order to further examine the stability of the *Metridia* luciferase signal, we transiently transfected the pNFκB-MetLuc2-Reporter Vector into HeLa cells. 24 hours later, we induced the cells with TNF-α to activate the NFκB signaling pathway. Six hours after that, we detected response element activation by assaying the cell supernatant for secreted *Metridia* luciferase activity. Although signal intensity after substrate addition decreased with time, the calculated overall fold induction was the same even 30 minutes after substrate addition (Figure 3; Haugwitz *et al.*, 2008).

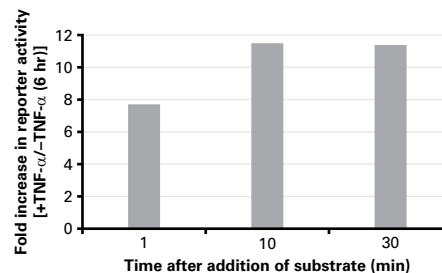


Figure 3. Monitoring promoter activation using the secreted *Metridia* luciferase reporter. HeLa cells were transiently transfected with a vector construct containing the NFκB response element driving the expression of sequence-optimized secreted *Metridia* luciferase. 24 hr after transfection, the media 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 time points following substrate addition.

I. Introduction continued

2. High Sensitivity & Broad Linear Range of the Secreted *Metridia* Luciferase Signal

We determined the sensitivity (limit of detection) of *Metridia* luciferase in standard mammalian cell culture conditions by spiking various amounts of purified protein into DMEM containing 10% FBS. Upon addition of the luciferase substrate, we were able to detect levels as low as 40 fg of recombinant *Metridia* luciferase per ml (2 fg per well in a 96-well plate). This corresponds to approximately 46,000 molecules. We then determined the linear range of *Metridia* luciferase activity by serial dilution of the recombinant protein, followed by substrate addition. *Metridia* luciferase activity is linear over a range of at least 6 logs (Figure 4; Haugwitz *et al.*, 2008).

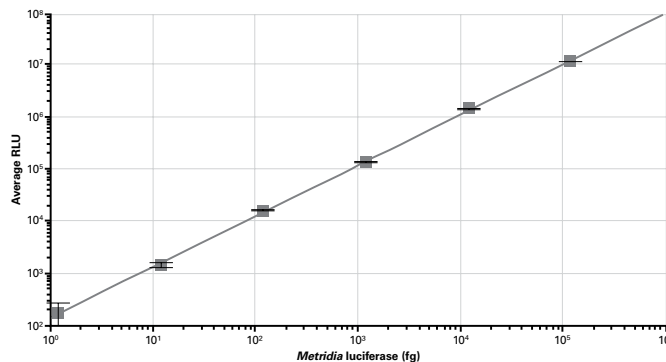


Figure 4. Limit of detection of recombinant *Metridia* luciferase activity. Various amounts of recombinant *Metridia* luciferase were spiked into DMEM + 10% FBS in a 96-well plate. After addition of substrate, the plate was analyzed using a Molecular Devices® SpectraMax® L plate reader.

D. The Ready-To-Glow Dual Secreted Reporter Assay

Multiplexing two different bioluminescent secreted reporters, using different substrates, provides even more information on the cell state without sacrificing the cells, and creates new advantages for experimental design and analysis. For an extended discussion about the SEAP reporter system, please refer to PT3057-1, the Great EscAPe™ SEAP Reporter System User Manual, which can be found at www.clontech.com/support/manuals.asp.

The **Ready-To-Glow Dual Secreted Reporter Assay** (No. 631734) uses two secreted reporters, *Metridia* luciferase and Secreted Alkaline Phosphatase (SEAP), that can be detected and distinguished from each other by reporter-specific substrates. When cells are cotransfected with both reporters and stimulated to express and secrete *Metridia* luciferase or SEAP, or both, promoter-specific activity can be detected in a sample of culture media by adding the relevant

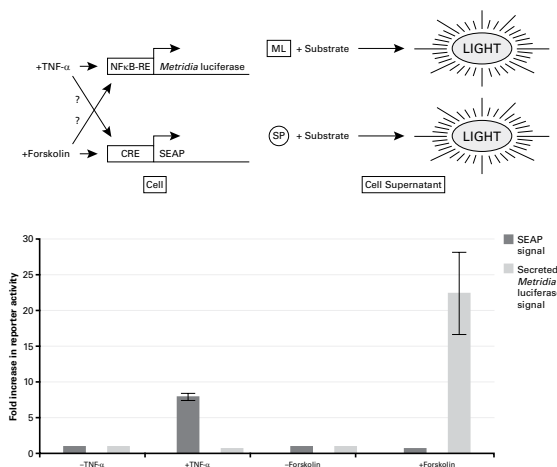


Figure 5. Monitoring the activities of two promoters using the Ready-To-Glow Dual Secreted Reporter System to investigate potential cross-talk between signaling pathways in the same cell. HEK 293 cells cotransfected with pNFκB-TA-MetLuc and pCRE-SEAP constructs were treated with fresh media alone, or with media containing either 1,000 ng/ml TNF-α or 10 μM forskolin. Media samples were collected from the transfected cells 7 hr later, and each sample was tested in triplicate. No cross talk was observed between the cAMP and NFκB signaling pathways.

I. Introduction continued

enzyme-specific substrate and measuring the resulting chemiluminescence. Because the reporters are secreted (and do not require cell lysis), you will be left with live cells that can be used repeatedly, either for multiple time point assays or for any other analysis on the cells.

Potential applications of the Ready-To-Glow Dual Secreted Reporter System include:

- Using one reporter as a transfection control for normalization purposes.
- Using one reporter as a positive, constitutive control to monitor the functionality of the secretory pathway.
- Monitoring two different signal transduction pathways simultaneously. This makes it possible to examine potential cross-communication between intracellular signal transduction pathways. For example, we used secreted *Metridia* luciferase and secreted alkaline phosphatase (SEAP) to determine whether the cAMP and NFκB signal transduction pathways are interrelated (Figure 5).

1. The Ready-To-Glow Dual Secreted Reporter Vectors

The **Ready-To-Glow Dual Secreted Reporter Vector Kit** (Cat. No. 631735) includes four vectors (e.g., two vector sets). It has been customized to work optimally with our Ready-To-Glow Dual Secreted Reporter Assay (Cat. No. 631734).

The reporter vectors are:

- **pMetLuc2-Reporter** contains an MCS that allows promoter or promoter/enhancer elements to be inserted upstream of the secreted *Metridia* luciferase (MetLuc) gene. Activation of the promoter/enhancer elements of interest drives the expression of secreted *Metridia* luciferase, which is detected in the supernatant of the transfected cells.
- **pSEAP2-Basic** lacks eukaryotic promoter and enhancer sequences and has an MCS that allows putative promoter DNA fragments to be inserted upstream of the SEAP gene. Enhancers can be cloned into either the MCS or unique downstream sites. Activation of the promoter/enhancer elements of interest drives the expression of SEAP, which is detected in the supernatant of the transfected cells.

Each reporter vector comes with a control vector (pMetLuc2-Control and pSEAP2-Control, respectively), which is designed to function as a positive control. The control vectors serve as positive controls for constitutive promoter activity in the luciferase- and SEAP-based assays respectively.

2. *Metridia* Luciferase and SEAP Secretion Kinetics Are Comparable

The *Metridia* luciferase and SEAP reporters are secreted from stable or transiently transfected cells into the culture medium, so you can sample repeatedly over time without sacrificing cells. Since the secretion kinetics of both reporters are very similar (Figure 6), the amount of either reporter in the cell culture medium accurately reflects its promoter's activity.

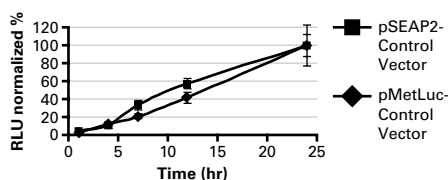


Figure 6. Similar secretion kinetics of *Metridia* secreted luciferase and SEAP enable accurate comparisons of the relative timing of promoter activity. HeLa cells were plated into 6-well plates and transiently transfected with either the pMetLuc-Control vector or the pSEAP-Control vector. Media samples from the transfected cells were collected from 3 wells at each time point by removing enough media to run either the luciferase or the SEAP assay. Each sample was tested in triplicate, using a white bottom 96-well microtiter plate on a Turner BioSystems Veritas Luminometer.

For examples of how the Ready-To-Glow Secreted Reporter Systems can be applied to your promoter studies, please see Appendix B.

II. Additional Materials Required

A. Microtiter Plates to Perform the Assay

Bioluminescence detection of luciferase activity can be performed using either a tube or plate luminometer when reactions are carried out in microcentrifuge tubes or in multiwell microtiter plates. Ready-To-Glow assays can be carried out either in 0.5 ml microcentrifuge tubes or multiwell microtiter plates. The following type of microtiter plate is recommended:

- **Microplate 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.

B. Vectors to Normalize Transfection Efficiency

The **Ready-To-Glow Dual Secreted Reporter Vector Kit (Cat. No. 631735)** includes the pSEAP2-Basic and -Control Vectors as well as the pMetLuc2-Reporter and -Control Vectors and the respective assays. The reporter vectors can be used as transcription reporters, while the control vectors serve as positive controls for constitutive promoter activity in the SEAP- (a secreted form of human alkaline phosphatase) and luciferase-based assays respectively. For both vector sets, promoter activity can be directly correlated to the amount of reporter (SEAP or luciferase) secreted in the medium.

Alternatively, any of the following vectors can be used to normalize transfection efficiency:

- Fluorescent protein vectors such as pAcGFP1-N1 Vector (Cat. No. 632469) or pDsRed-Express2-N1 Vector (Cat. No. 632537).
- pSEAP2-Control Vector (sold separately as Cat. No. 631717)
- pCMVβ Vector (Cat. No. 631719)

C. *E. coli* Competent Cells for Plasmid Propagation

Either of the following competent *E. coli* strains can be used for plasmid propagation:

- Stellar™ Competent Cells (Cat. No. 636763)
- Stellar Electrocompetent Cells (Cat. No. 636765)

D. Kits for Plasmid DNA Isolation

For rapid, high yield isolation of transfection grade plasmid DNA, we recommend using NucleoBond Xtra Kits:

- NucleoBond Xtra Midi Plus Kits (Cat. Nos. 740412.50 & 740412.50)
- NucleoBond Xtra Maxi Plus Kits (Cat. Nos. 740416.10 & 740416.50)

E. Sequencing Primers

- We recommend using the following primer (not available from Clontech) to sequence inserts cloned into the pMetLuc2-Reporter Vector. This reverse primer can be used to sequence from the 5' region of the *Metridia* reporter gene region into the upstream MCS:

5'-CACGATGTCGATGTTGGGG-3'
(183–165 in pMetLuc2-Reporter Vector)

- We recommend the following primer (not available from Clontech) to sequence from the 5' region of the SEAP reporter gene region into the MCS:

5'-CCTCGGCTGCCTCGCGGTTCC-3'
(376–356 in pSEAP2-Control)

- We recommend the following primer (not available from Clontech) to sequence inserts cloned into the pLVX-MetLuc2-Reporter Vector:

5'-ATGCCAACTTGCTTCCAGGCC-3'
(2333–2356 in pLVX-MetLuc2-Reporter Vector)

III. Ready-To-Glow Protocols

PLEASE READ THROUGH THE ENTIRE PROTOCOL BEFORE BEGINNING.

A. Sample Preparation

1. Transfect your cells of interest with the secreted *Metridia* luciferase reporter vector of your choice. You can perform either a transient or stable transfection, using your transfection method of choice. For information about transfections, please see Appendix A.
2. Incubate your cells for the amount of time your transfection protocol recommends for optimal transfection efficiency.
3. Remove the culture media and split the cells to a multiwell plate, following standard tissue culture protocols. Alternatively, you can leave the cells in the culture plate for your experiment.
4. At the beginning of your experiment (i.e., when you begin your experimental treatment) remove the culture media and replace it with fresh media containing the candidate promoter inducer/inhibitor of your choice.

NOTE: Changing the tissue culture media eliminates any background signal caused by the accumulation of secreted *Metridia* luciferase in the supernatant before the start of the experiment.

5. Collect 50 μ l samples of media supernatant at different time points after starting the treatment. How long you should wait before collecting samples depends on your particular experiment. We recommend that you begin by collecting media samples 2, 8, 12, and 24 hours after the start of the experiment.
6. You can assay the media samples for secreted luciferase activity immediately, or freeze them for later analysis. If multiple samples are collected, they can be transferred into a 96-well plate for further analysis (See Section II for recommended plates).



III. Ready-To-Glow Protocols continued



**Protocol
15 min.**

B. Protocol for Non-Automated *Metridia* Luciferase Assays

1. Prepare the 10X Substrate Stock Solution.

Dissolve the Lyophilized Secreted Luciferase Substrate by adding Substrate Buffer:

<u>Number of Reactions</u>	<u>Quantity of Substrate Buffer</u>
100 rxns (Cat. No. 631726)	60 μ l
500 rxns (Cat. No. 631727)	250 μ l
1,000 rxns (Cat. No. 631728)	500 μ l



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. A 15 min incubation at room temperature (RT) can accelerate the reconstitution.

Store the 10X Substrate Stock Solution at -20° C. We do not recommend aliquoting the solution, as this may induce oxidation.

2. Prepare the 1X Substrate/Reaction Buffer.

Dilute the 10X Substrate Stock Solution (Step III.B.1) 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 a factor of 5.

For example, for 20 samples, you would prepare $20 \times 5 = 100$ μ l of 1X Substrate/Reaction Buffer by diluting 10 μ l of 10X Substrate Stock Solution in 90 μ l of Reaction Buffer.



TO ENSURE CONSISTENT RESULTS, prepare fresh 1X fresh Substrate/Reaction Buffer every time, without introducing air bubbles. Slowly pipet the Substrate Stock Solution or use 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 the colorless Reaction Buffer, the resulting 1X solution is light yellow/orange in color. Allow the 1X Substrate/Reaction Buffer to remain at room temperature for 10 min before use. It can remain at room temperature for up to 1 hr before use.



3. Transfer 50 μ l of culture media from each sample (Step III.A.5) into one well of a 96 well plate (please see Section II for a 96 well-plate recommendation).

NOTE: For statistical reasons, we recommend assaying triplicates of each sample.

4. Add 5 μ l of 1X Substrate/Reaction Buffer (Step III.B.2) to each sample.
If you have many samples in a 96-well plate, we recommend using a multichannel pipette in order to reduce the time between substrate addition and signal detection.
5. Transfer the plate to a luminometer and record light signals according to the manufacturer's recommended luminometer settings.

TIP: We recommend reading the plate(s) immediately, as well as 5 and 10 min after substrate addition. 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.

III. Ready-To-Glow Protocols continued



**Protocol
15 min.**



C. Protocol for Automated *Metridia* Luciferase Assays

1. Prepare the 100X Substrate Stock Solution:

<u>Number of Reactions</u>	<u>Quantity of Substrate Buffer</u>
1,000 rxns (Cat. No. 631739)	500 µl
5,000 rxns (Cat. No. 631740)	500 µl/ vial (2.5 ml in total, for 5 vials)

NOTE: 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. A 15 min incubation at room temperature (RT) can accelerate the reconstitution.

Store the 100X Substrate Stock Solution at -20°C . We do not recommend aliquoting the solution, as this may induce oxidation.

2. Prepare the 1X Substrate/Reaction Buffer:

Dilute the 100X Substrate Solution (Step III.C.1) 1:100 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 a factor of 50.

For example, for 100 samples, you would prepare $100 \times 50 = 5,000$ µl of 1X Substrate/Reaction Buffer by diluting 50 µl of 100X Substrate Stock Solution in 4,950 µl of Reaction Buffer.



TO ENSURE CONSISTENT RESULTS, prepare fresh 1X fresh Substrate/Reaction Buffer every time, without introducing air bubbles. Slowly pipet the Substrate Stock Solution or use 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 the colorless Reaction Buffer, the resulting 1X solution is light yellow/orange in color. Allow the 1X Substrate/Reaction Buffer to remain at room temperature for 10 min before use. It can remain at room temperature for up to 1 hr before use.



3. Transfer 50 µl of culture media from each sample (Step III.A.5) into one well of a 96 well plate (please see Section II for a 96 well-plate recommendation).

NOTE: For statistical reasons, we recommend assaying triplicates of each sample.

4. Add 50 µl of 1X Substrate/Reaction Buffer (Step III.C.2) to each sample, either by pipetting it into the wells manually or via an automatic injector system connected to your plate reader.

TIP: Do not vortex the plate to mix the sample and substrate. If you have many samples, we recommend using a multichannel pipette in order to reduce the time between substrate addition and signal detection.

5. Transfer the plate to a luminometer and record light signals according to the manufacturer's recommended luminometer settings.

TIP: We recommend reading the plate(s) immediately, as well as 5 and 10 min after adding the substrate.

III. Ready-To-Glow Protocols continued

D. Chemiluminescent SEAP Reporter Assay (Dual Secreted Reporter System Only)

For transient transfection assays using the pSEAP2-Control vector (Sold as part of the Ready-To-Glow Dual Secreted Reporter Vector Kit, Cat. No. 631735), the secreted form of human alkaline phosphatase (SEAP) is generally detected in the medium 12–18 hours after transfection, with maximal levels detected between 48–72 hours. Optimal times will vary depending on the cell type, cell density, and the particular experimental conditions. Each construct should be transfected and assayed in triplicate.

1. Prepare Reagents and Samples for the SEAP Assay

- a. Transfer 25 μ l of cell culture medium from transfected cells or mock transfected cells (in triplicate) to a 96-well microtiter plate. If necessary, the plate can be frozen at -20°C for future analysis.

NOTE: We recommend Microlite 1 Luminescence Microtiter 96-well plates (VWR Scientific Products, Cat. No. 62403-124).

- b. Prepare 1X Dilution Buffer by diluting the 5X Dilution Buffer 1:5 with ddH₂O.

2. Perform the SEAP Assay

You may need to dilute some samples in order to stay within the linear range of the assay. To determine the linear range, assay a dilution series of your sample and a dilution series of recombinant human placental secreted alkaline phosphatase before you assay your samples.

- a. Allow the SEAP Substrate Solution to equilibrate to room temperature ($22\text{--}25^{\circ}\text{C}$).
- b. Add 75 μ l of 1X Dilution Buffer to each sample (from Step 1.a.) in the 96-well microtiter plate.
- c. Seal the plate with adhesive aluminum foil or a 96-well plate lid and incubate the diluted samples for 30 min at 65°C using a heat block or water bath.
- d. Cool the samples on ice for 2–3 min, then equilibrate to room temperature.
- e. Add 100 μ l of SEAP Substrate Solution to each sample. Incubate for 10–60 min (30 min recommended) at room temperature before reading.
- f. Use a 96-well plate reader luminometer to detect and record the SEAP signal. Optimal readings will be obtained 10–60 min after substrate addition.

Refer to your plate reader's user manual for additional information regarding its performance and use.



IV. Troubleshooting Guide

Problem	Explanation	Solution
Little or no signal from transfected cells.	Number of transfected cells is too low.	Ensure that the transfection efficiency has been optimized by using the supplied control vector as an internal positive control for luciferase expression.
		Increase the number and/or density (or concentration) of cells used in transfections.
	Transfected cells are not yet producing <i>Metridia</i> luciferase.	If the signal from the positive control (media from cells transfected with the pMetLuc-Control Vector) is low, incubate the transfected cells in media for a longer time before collecting supernatant samples.
		Increase the time of transfection before starting the experiment.
	Plate reader requires adjustment.	Refer to the instrument's instructions for methods to increase the sensitivity of light detection.
Signal quenches rapidly (within a few minutes of substrate addition).	Although the signal intensity decreases over time, the overall fold induction remains the same for ~30 min after substrate addition (Part I, Figure 3).	Continue with your experiment as usual.
Substrate stock solution darkens over time.	Possibly due to partial oxidation of the substrate.	Ensure that the substrate is working properly (using appropriate positive and negative controls) before assaying experimental samples.
High background signal.		Decrease the volume of media assayed from experimental cultures. Alternatively, dilute your samples.
Signal exceeds the linear range of the assay.		Dilute your samples, using the same media that was used to culture the cells.
		If you are in doubt about the linear range of the assay, prepare and assay a dilution series using the positive control (media from cells transfected with the pMetLuc-Control Vector).

IV. Troubleshooting Guide continued

Table II. Troubleshooting the SEAP Assay* (Dual Ready-To-Glow System Only)		
Problem	Explanation	Solution
Little or no signal from transfected cells.	Number of transfected cells is too low.	Ensure that the transfection efficiency has been optimized by using pSEAP2-Control as an internal positive control for SEAP expression.
		Increase the number and/or density (or concentration) of cells used in transfections.
		Increase the post-transfection interval prior to collecting media samples.
		If background signals from negative controls (i.e., cells transfected with pSEAP2-Basic) are low, increase the volume of media assayed from experimental cultures from 25 μ l to 50–75 μ l (adjust the 1X Dilution Buffer volume accordingly.)
	Problem with assay conditions.	Ensure that the assay conditions are correct and that the detection method is working by assaying the positive control enzyme. Ensure that the conditioned media does not contain an inhibitory activity by adding 2 μ l of Positive Control Placental Alkaline Phosphatase to 25 μ l of culture medium
	Plate reader requires adjustment.	Refer to the instrument's instructions for methods to increase the sensitivity of light detection.
High background signal.		Ensure that the diluted media samples are heated for the full 30 min at 65°C as specified in Step III.D.2.c.
		Decrease the volume of media assayed from experimental cultures. Alternatively, dilute your samples. Note: If diluting with fresh serum-containing media, please ensure heat inactivation of any endogenous alkaline phosphatase by incubation at 65°C for 30 min.
		If possible, after transfection grow cells in media containing minimal serum. Serum levels >10% (v/v) may increase background.
Signal exceeds 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 Placental Alkaline Phosphatase.
		Decrease the volume of media assayed from experimental cultures. Alternatively, dilute your samples. Note: If diluting with fresh serum-containing media, please ensure heat inactivation of any endogenous alkaline phosphatase by incubation at 65°C for 30 min.
		Perform a time course after induction by collecting and assaying media supernatant at different time points to find a suitable time point, at which the signal falls in the linear range.

*For an extended discussion about the SEAP reporter system, please refer to PT3057-1, the Great EscAPe SEAP Reporter System User Manual, which can be found at www.clontech.com/support/manuals.asp.

V. References

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Appendix A. Transfections & Experimental Controls

A. Transfections

1. Transfection Methods:

All Ready-To-Glow vectors can be transfected into mammalian cells by your standard transfection method of choice. If you do not have an established transfection protocol or if you are working with a cell line for the first time, we recommend comparing different protocols, using the pMetLuc2-Control vector and monitoring the amount of secreted *Metridia* luciferase in the cell supernatant, in order to determine efficiency and choose an appropriate protocol.

2. Transfection Considerations:

- a. **Perform each transfection in triplicate.** If cells are sampled in multiwell plates, we recommend transfecting one 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 second reporter can then be used to normalize the levels of luciferase among different treatment groups and different transfections.

The **Ready-To-Glow Dual Secreted Reporter Vector Kit (Cat. No. 631735)** includes the pSEAP2-Basic and -Control Vectors as well as the pMetLuc2-Reporter and -Control Vectors and the respective assays. The reporter vectors can be used as transcription reporters, while the control vectors serve as positive controls for constitutive promoter activity in the SEAP- (a secreted form of human alkaline phosphatase) and luciferase-based assays respectively. For both vector sets, promoter activity can be directly correlated to the amount of reporter (SEAP or luciferase) secreted in the medium.

Other reporter proteins frequently used for normalization include SEAP (the pSEAP2-Control Vector, sold separately as Cat. No. 631717), *E. coli* β -galactosidase (pCMV β Vector, Cat. No. 631719), and fluorescent protein vectors such as the pAcGFP1-N1 Vector (Cat. No. 632469) or the pCMV-DsRed-Express Vector (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. To determine the background level, perform the assay using 50 μ l samples of culture medium from mock-transfected cells (without vector). The values obtained from such controls should be subtracted from experimental results.

2. Positive Controls:

A positive control is necessary to confirm transfection and expression of exogenous DNA, and to verify that your cells are secreting *Metridia* luciferase into the culture media.

To confirm the expression and secretion of functional luciferase in transfected cells, perform the assay using 50 μ l of culture medium from cells transfected with the pMetLuc2-Control Vector, which contains the luciferase gene under transcriptional control of the constitutive CMV promoter. Cells transfected with this plasmid should yield high activity within 12–36 hours (often even earlier) after transfection.

3. Normalizing Transfection Efficiencies:

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

Appendix B. Example Applications

A. High-Throughput (HT) Applications

The Ready-To-Glow Secreted Luciferase System is a powerful tool for HT applications because it is easy to use, eliminates cell lysis, and guarantees high sensitivity and a broad linear range. It has been used successfully in a 1,536-well format to visualize the expression of secreted *Metridia* luciferase, even using a regular, low-sensitivity CCD camera (Figure 7). In addition, the assay can be performed in the presence of cells by adding the substrate directly to the culture media.

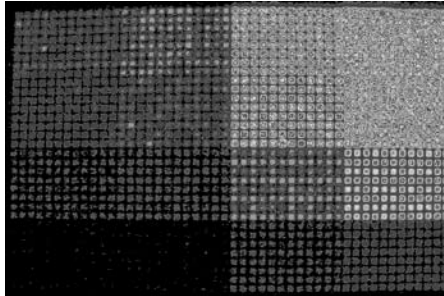


Figure 7. Use of secreted *Metridia* luciferase in a High-Throughput Screening (HTS) application. This is a screen shot of a 1,536-well plate containing stable CHO cells transfected with a forskolin-responsive *Metridia* luciferase gene (pASM-Lu164), taken with a CCD camera. 300 cells/well were plated in a 1,536-well microtiter plate. Cells were incubated with increasing concentrations of forskolin and the plate was visualized using a CCD camera system (integration time: 60 sec). This pseudocolor image reflects luminescence intensity. Near white and gray regions are the brightest, while black regions are the least bright. Courtesy of Bayer Health Care, Germany.

B. Multiplexing Bioluminescent Secreted Reporters & Fluorescent Reporters

Plate readers that can detect bioluminescent *and* fluorescent reporter activity at the same time, in the same well create opportunities to develop new multiplex assays that are unlimited by the method of detection. When multiplexing a secreted reporter and a fluorescent reporter, there is no requirement to sacrifice the cells—expanding the possibilities even more.

We used this type of multi-mode, multiplexing approach to determine the role of the proteasomes in TNF- α -induced, NF κ B-dependent signaling. We used the Living Colors® Proteasome Sensor HEK 293 Cell Line to monitor proteasomal activity: if the proteasomes are active, only a very low level of green fluorescence is observed in the cytosol of this cell line. However, if the function of the cellular proteasomes is compromised, there is a sharp rise in the fluorescent intensity in the cytosol of this stable cell line. At the same time, we used secreted *Metridia* luciferase to monitor NF κ B-based promoter activation.

Using these two reporters, we were able to determine the importance of proteasomal activity in the NF κ B dependent, TNF- α -induced signaling pathway. It has been shown previously that phosphorylation of I κ B alone upon TNF- α treatment is not sufficient to allow NF κ B-induced promoter activation (Haas *et al.*, 1998; Sun & Carpenter, 1998). Consistent with these findings, our results show that phosphorylation of I κ B must be followed by its proteasomal degradation, in order to allow NF κ B-based promoter activation (Figure 8; Haugwitz *et al.*, 2008). This experiment demonstrates the power of multiplexing bioluminescent and fluorescent reporters in a live cell assay: we were able to detect two events simultaneously, from the same well of a 96-well plate, simply by collecting both fluorescent and bioluminescent signals using a multi-mode plate reader.

Appendix B. Example Applications continued

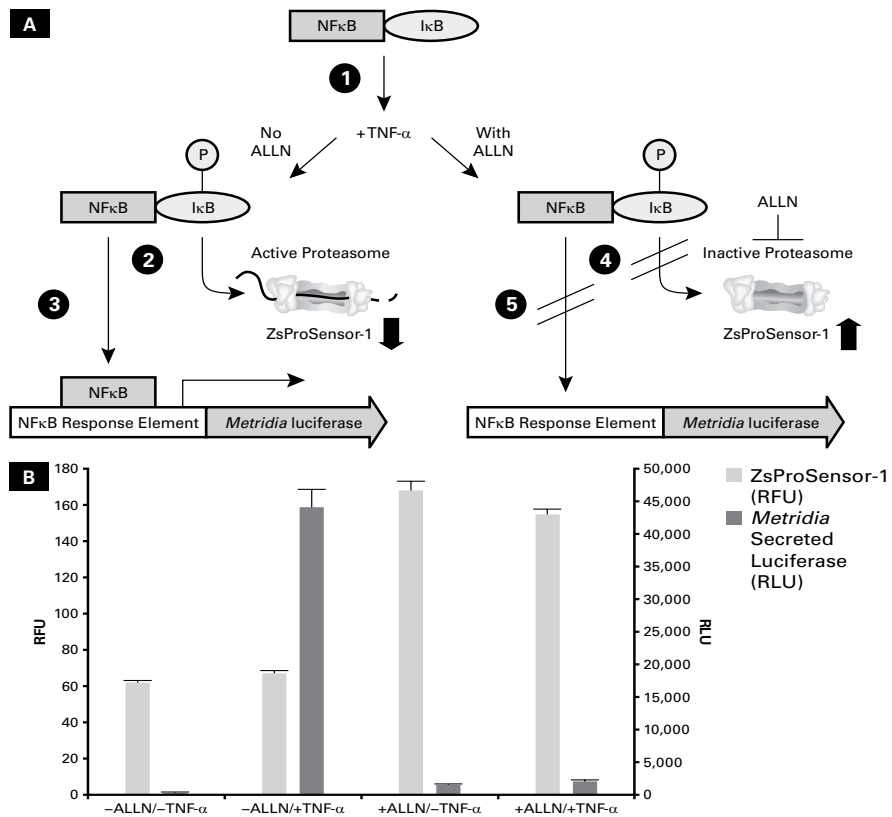


Figure 8. Multiplexing bioluminescent secreted reporters and fluorescent reporters to determine the requirement for active proteasomes in TNF- α -induced, NF κ B-dependent signaling. Panel A. Inactive NF κ B is sequestered in the cytoplasm by I κ B. I κ B must be phosphorylated upon TNF- α induction (1) and degraded by the proteasome (2) in order for NF κ B to translocate to the nucleus and initiate signaling (3). Alternatively, when the proteasome is inhibited by the peptide ALLN (4), I κ B is not degraded and NF κ B cannot translocate (5). The status of the proteasome (active or inactive) can be monitored based on ZsProSensor-1 levels. **Panel B.** NF κ B activation by TNF- α requires proteasomal activity. High levels of *Metridia* luciferase signal were only observed in the absence of ALLN and the presence of TNF- α . When the proteasomes were inactivated by ALLN (indicated by increasing levels of ZsProSensor-1 fluorescence), the NF κ B signaling pathway could not respond effectively to TNF- α stimulation.

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