

Ultrasensitive Secreted Luciferases and their use in a Dual Reporter System

Introduction to *Gaussia* and *Cypridina* Luciferases

Luciferases are enzymes that produce light as a by-product of the oxidation of substrate. This light emission can be easily detected, making luciferases ideal reporter systems. In fact, they have proven to be very useful reporters for the study of fundamental cellular processes such as the regulation of transcription and translation, mRNA and protein stability, nucleic acid and protein interactions, as well as markers of cellular physiology and responses.

The first luciferase reporters used in life science research were intracellular luciferases. These non-secreted reporters require that cells be lysed in order to measure activity. Measurement of reporter activity at a number of different time points involve complex experimental design with large numbers of wells and reagents. Additionally, the fact that each time point is taken from different cell samples can contribute to experimental variability.

The availability of secreted luciferase proteins overcomes these difficulties. New England Biolabs has recently developed reporter systems based on *Gaussia* luciferase (GLuc) and *Cypridina* luciferase (CLuc) (1,2). These luciferases produce a large number of photons per reduction cycle, making them very sensitive reporters. When expressed in mammalian cells, GLuc and CLuc are naturally secreted into the growth media (Fig. 1) (3), allowing the user to sample luciferase activity from the same source multiple times over the course of an experiment. This method of non-destructive sample collection expedites the assay protocol, simplifies experimental design and allows the cells to remain intact so they can be used in further downstream assays such as RT-PCR, Western blots, RNA expression analysis, live imaging, cell viability assays, etc.

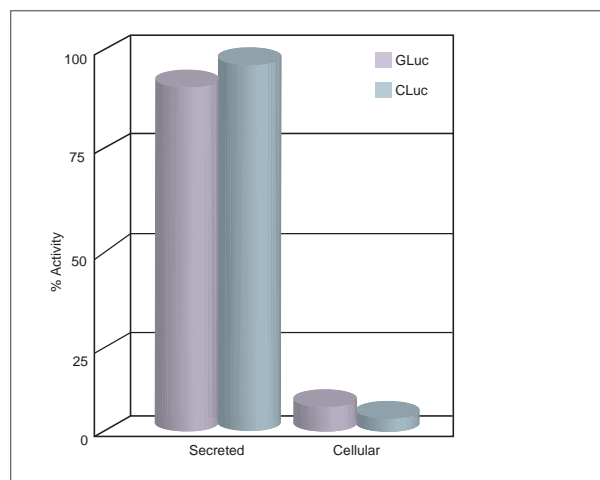


Figure 1: Both *Gaussia* and *Cypridina* luciferases are efficiently secreted from mammalian cells. GLuc and CLuc vectors were transfected into CHO-K1 cells. Supernatants were collected and cell lysates were made. Luciferase activity was measured from 20 μ l of supernatant or lysate from the same cells and the percentage of total luciferase activity in the supernatant (secreted) and in the lysate (cellular) was calculated. Activity of GLuc and CLuc was measured with its corresponding BioLux assay system.

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GENE EXPRESSION & CELLULAR ANALYSIS

General Protocol

Injector-equipped luminometers

1. Prepare the GLuc assay solution (e.g. 100 samples) by adding 50 μ l of BioLux™ GLuc Substrate to 5 ml of Bioluminescence Assay Buffer immediately before performing the assay. (Be sure to prepare enough assay solution as needed for all samples as well as for priming a particular luminometer as recommended by the manufacturer).
2. Mix well by inverting the tube several times (Do not vortex).
3. Set the luminometer with the following parameters: 50 μ l of injection, 5–15 seconds of delay & 2–10 seconds of integration.
4. Pipet samples (5–20 μ l per well) into a 96-well white (opaque) or black plate, or a luminometer tube.
5. Prime the injector with the GLuc assay solution and proceed with the measurement.

* Approximately 90% of GLuc is secreted out into the growth media after transfection and thus, the GLuc activity is typically assayed from the supernatant (i.e. growth media of GLuc-transfected cells). However, as long as the cells are alive, approximately 10% of GLuc is present inside the cells. Therefore, GLuc activity can also be assayed from the cell lysate. We recommend that the cell lysates be prepared by using Luciferase Cell Lysis Buffer (NEB #B3321), since this lysis buffer is designed to be compatible with *Cypridina*, *Gaussia*, *Renilla*, Firefly luciferase and β -gal activity assays.

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Use of GLuc and CLuc in a Dual Reporter System

The co-transfection of an experimental reporter and a second control reporter can provide valuable information, including tracking of a reporter gene of interest, transfection efficiency and cell viability. The transfection efficiency of the secondary reporter serves as a normalizer and allows researchers to compare results within and across experiments. To take full advantage of the benefits of a secreted experimental reporter, it is best to also use a secreted control reporter, allowing the user to continuously monitor the secretion of both transfected reporters into the growth media. Additionally, it minimizes sampling error, as both reporters can be measured from a fraction of the same sample.

GLuc and CLuc utilize different substrates to carry out the light emitting oxidation reaction, and both exhibit a similar range of activity (4). Figure 2 shows that the detection of one luciferase can be monitored without any cross-reactivity from the presence of the other. Briefly, supernatants were collected from CLuc- and GLuc-expressing cells and mixed, maintaining the fraction of one supernatant constant while increasing the volume of the other supernatant. Activity of GLuc and CLuc in the mixture was measured with its corresponding BioLux assay system. These results demonstrate that GLuc and CLuc are ideal reporter partners to be used in co-expression experiments in mammalian cells.

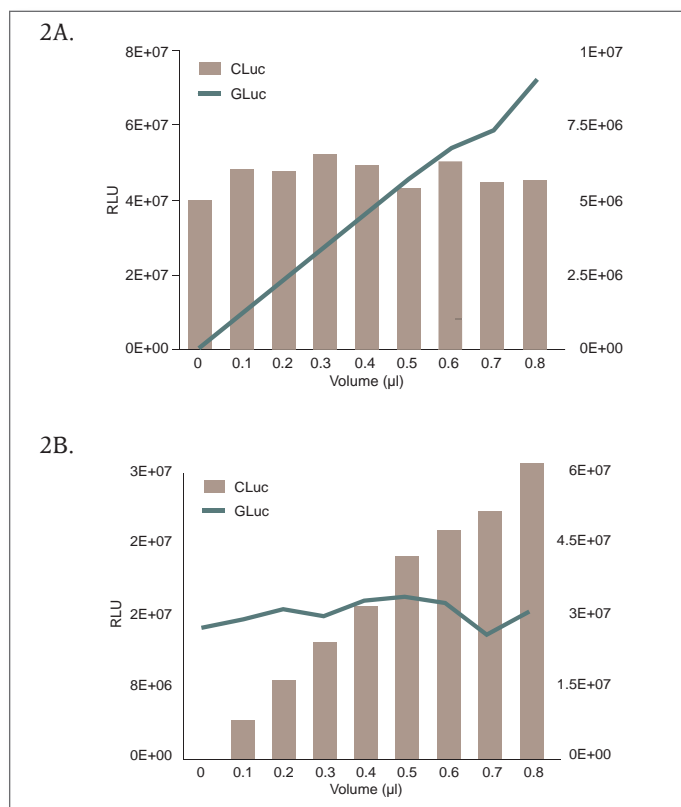


Figure 2: GLuc or CLuc activity can be measured without interference from the presence of the other luciferase. (A) supernatant from CLuc cells is held constant while volume of supernatant from GLuc cells is increased. (B) supernatant from GLuc cells is held constant while volume of supernatant from CLuc cells is increased. Activity is measured with the corresponding BioLux Assay System.

References

1. Verhaegen, M. and Christopoulos, T.K. (2002) *Anal. Chem.* 74, 4378-4385.
2. Nakajima, et al. (2004) *Biosci. Biotechnol. Biochem.* 68, 565-570.
3. Knappskog, S., et al. (2007) *J. Biotechnol.*, 128, 705-715.
4. Wu, C., Suzuki-Ogoh, C. and Ohmiya, Y. (2007) *Biotechniques*, 42, 290-292.

General Protocol

Luminometers without injectors

1. Prepare the GLuc assay solution (e.g. 100 samples) by adding 50 μl of BioLux GLuc Substrate to 5 ml of BioLux GLuc Assay Buffer immediately before performing the assay.
2. Mix well by inverting the tube several times (Do not vortex).
3. Set the luminometer for 2-10 seconds of integration.
4. Pipet samples (5-20 μl per well) into a 96-well white (opaque) or black plate, or a luminometer tube.
5. Add the GLuc assay solution (50 μl) to a sample (i.e. Add the assay solution to only one sample at a time) and promptly measure the luminescence.
6. Repeat Step 5 for all samples.

The Sensitivity and Stability of Secreted Luciferases from New England Biolabs

Introduction

Luciferases are enzymes that produce light as a by-product of the oxidation of a substrate. This light emission can be easily detected, making luciferases ideal reporter systems. In fact they have proven to be very useful reporters for the study of fundamental cellular processes such as transcriptional and translation regulation, mRNA and protein stability, nucleic acid and protein interactions, as well as markers of cellular physiology and responses.

Sensitivity

New England Biolabs currently offers two reporter systems that utilize secreted luciferases: *Gaussia* Luciferase (GLuc) isolated from the copepod *Gaussia princeps*, and NEB *Cypridina* Luciferase (CLuc) isolated from the ostracod *Cypridina noctiluca*. These are the brightest luciferases commercially available (1-3). Their brightness allows the user to detect activity in small samples or from cells with low levels of reporter gene expression. GLuc and CLuc are naturally secreted by mammalian cells into the growth media (4). Although the majority of the luciferase produced by the transfected cells is secreted, the sensitivity of these systems also enables the user to detect luciferase activity in lysates (Fig. 1).

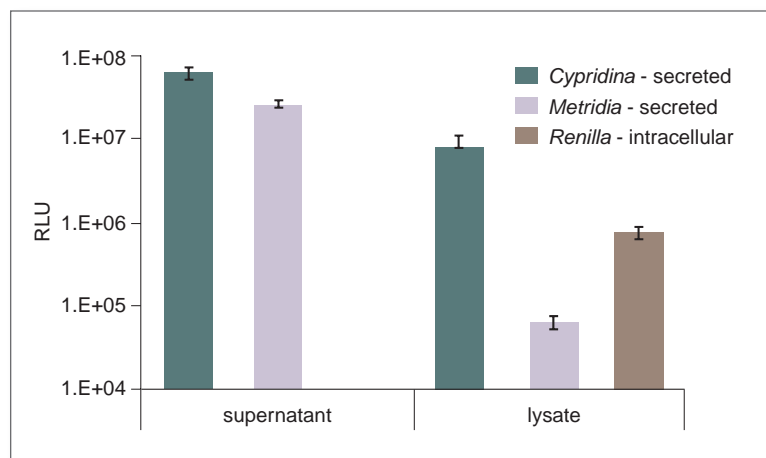


Figure 1: Sensitivity of *Cypridina* Luciferase compared to other reporters: *Cypridina* Luciferase activity is higher than *Metridia*, another secreted luciferase, and *Renilla*, a standard intracellular luciferase. Supernatant was collected or lysates were made from cells transfected with either a CMV-CLuc, CMV-*Metridia*- or CMV-*Renilla*-expressing vector. 20 μ l of supernatant (out of 500 μ l total) or lysate (out of 100 μ l total) were used to measure luciferase activity. Each luciferase was measured with its corresponding assay system.

(See other side)

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General Protocol

Luminometers without injectors

1. Thaw BioLux *Cypridina* Luciferase Assay Buffer (1X) completely to room temperature (protect from light) and mix well before use.
2. Prepare the CLuc assay solution (e.g. for 100 samples add 50 μ l of the reconstituted substrate (100X solution) to 5 ml of BioLux *Cypridina* Luciferase Assay Buffer).
3. Mix well by inverting the tube several times (do not vortex).
4. Incubate at room temperature for 30 minutes (protect from light).
5. Set the luminometer for 2–10 seconds of integration.
6. Pipet samples *(5–20 μ l per well) into a 96-well white (opaque), black plate or cuvette.
 - * Approximately 90% of *Cypridina* Luciferase is secreted into the culture medium after transfection and thus, the CLuc activity is typically assayed in the supernatant (i.e. culture medium of CLuc-transfected cells). However, as long as the cells are alive, ~10% of CLuc is present inside the cell and therefore, the CLuc activity can also be assayed in the cell lysate (Figure 7). We recommend that the cell lysates be prepared by using Luciferase Cell Lysis Buffer (NEB #B3321), since this lysis buffer is designed to be compatible with *Cypridina*, *Gaussia*, *Renilla*, Firefly Luciferase and β -gal activity assays.
7. Add the CLuc assay solution (50 μ l) to a sample (i.e. add the assay solution to only one sample at a time) and promptly proceed with the measurement.
8. Repeat Step 7 for all samples.

The combination of the properties of these luciferases with our optimized detection assays results in the ability to detect the activity with a remarkable range of sensitivity. For example, Figure 2 shows that it is possible to dilute conditioned medium 10,000 fold and still detect robust luciferase activity.

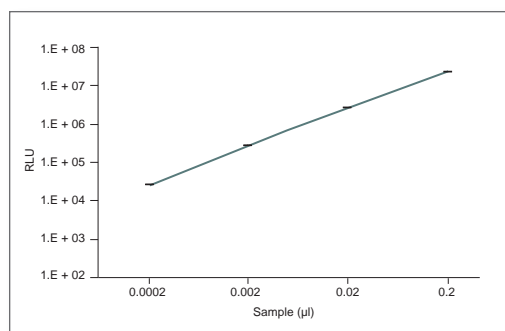


Figure 2: *Cypridina* luciferase activity can be measured in samples diluted 10,000 fold. Twenty µl of supernatant obtained from CLuc-expressing cells was diluted in a 10-fold serial dilution series and the CLuc activity was measured using the BioLux CLuc assay system.

Stability

Both GLuc and CLuc are very stable proteins. Their proper folding and activity requires the formation of a number of disulfide bonds at least (four and seventeen, respectively)(1), which confers stability at high temperatures and under strong acidic and basic conditions. This feature makes luciferase reporter systems more versatile, as they can be used under conditions that render other luciferases inactive. Both proteins maintain most of their activity at 55°C, which is the typical temperature to inactivate most viruses in samples (Fig. 3), and in the presence of β-mercaptoethanol, a standard additive to mES cell culture media.

The stability of these luciferases also provides the user with added flexibility. These secreted luciferases are stable in the growth media, so their activity accurately reflects the amount of protein accumulated in the supernatant up to the time of sampling. Furthermore, supernatants and lysates containing GLuc or CLuc can be stored for extended periods of time at -20°C and the proteins are stable through several freeze-thaw cycles.

The range of detection sensitivity combined with the stability of the protein and the kinetic properties of the light emitting reaction make these luciferases ideal for their use in high throughput reporter assays.

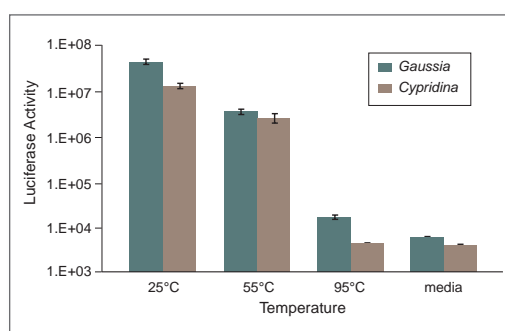


Figure 3: Both GLuc and CLuc are stable at different temperatures. Supernatants obtained from GLuc- or CLuc-expressing cells were incubated at the indicated temperature for fifteen minutes. Supernatants were then cooled to room temperature and assayed for luciferase activity. Each luciferase was measured with its corresponding BioLux assay system.

References

- Goerke, A., Loening, A., Gambhir, S. and Swartz, J. (2008) *Metabolic Engineering*, 10, 187–200.
- Tannous, B.A., et al. (2005) *Mol. Ther.*, 11, 435–443.
- Wu, C., Suzuki-Ogoh, C. and Ohmiya, Y. (2007) *Biotechniques*, 42, 290–292.
- Knappskog, S., et al. (2007) *J. Biotechnol.*, 128, 705–715.

General Protocol

Injector-equipped luminometers

1. Thaw BioLux *Cypridina* Luciferase Assay Buffer (1X) completely to room temperature (protect from light) and mix well before use.
2. Prepare the CLuc assay solution (e.g. for 100 samples add 50 µl of the reconstituted substrate (100X solution) to 5 ml of BioLux *Cypridina* Luciferase Assay Buffer).

Note: Be sure to prepare enough CLuc assay solution as needed for all samples as well as for priming the injector as suggested by the manufacturer.

3. Mix well by inverting the tube several times (do not vortex).
4. Incubate at room temperature for 30 minutes (protect from light).
5. Set the luminometer with the following parameters: 50 µl injection, 1–2 seconds of delay and 2–10 seconds of integration.

6. Pipet samples *(5–20 µl per well) into a 96-well white (opaque) or black plate.

* Approximately 90% of *Cypridina* Luciferase is secreted into the culture medium after transfection and thus, the CLuc activity is typically assayed in the supernatant (i.e. culture medium of CLuc-transfected cells). However, as long as the cells are alive, ~10% of CLuc is present inside the cell and therefore, the CLuc activity can also be assayed in the cell lysate (Figure 7). We recommend that the cell lysates be prepared by using Luciferase Cell Lysis Buffer (NEB #B3321), since this lysis buffer is designed to be compatible with *Cypridina*, *Gaussia*, *Renilla*, Firefly Luciferase and β-gal activity assays.

7. Prime the injector with the CLuc assay solution and proceed with the measurement.