



REPORTER SYSTEMS

BioLux[®]
Cypridina Luciferase Starter Kit

Instruction Manual

NEB #E3314S
100 assays

 NEW ENGLAND
BioLabs[®] Inc.
enabling technologies in the life sciences

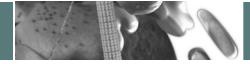


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Kit Includes:

pSV40-CLuc Control Plasmid	20 µg
pCLuc-Basic 2 Vector	20 µg
BioLux <i>Cypridina</i> Luciferase Assay Buffer (1X)	5 ml
BioLux <i>Cypridina</i> Luciferase Substrate Solvent [†]	0.5 ml
BioLux <i>Cypridina</i> Luciferase Substrate	lyophilized powder

(Kit components are not sold separately)

[†] The BioLux *Cypridina* Luciferase Substrate Solvent must be completely thawed and diluted with absolute ethanol (not included) before using it to make the 100X substrate solution (please refer to the section of Reconstitution of BioLux *Cypridina* Luciferase Substrate).



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U.S. Patent Nos. 7,718,389; 7,989,621; 8,367,357 and 8,343,729

Japanese Patent Nos. 4,761,150 and 4,484,429

Japanese Appln. Serial No.: 2006-280827; 2007-536587 and 2009-257631

EPO Appln. Serial No.: 06 810 525.3

Chinese Appln. Serial No.: 200680035410.3

For use of the BioLux *Cypridina* Luciferase Assay Kit, or associated assay reagents, in human diagnosis and measurement in relation to human health, contact busdev@neb.com.

Storage Information:

The BioLux *Cypridina* Luciferase Starter Kit should be stored at -20°C .

IMPORTANT:

BioLux *Cypridina* Luciferase Assay Buffer (1X) and Substrate (lyophilized powder) must always be protected from light.

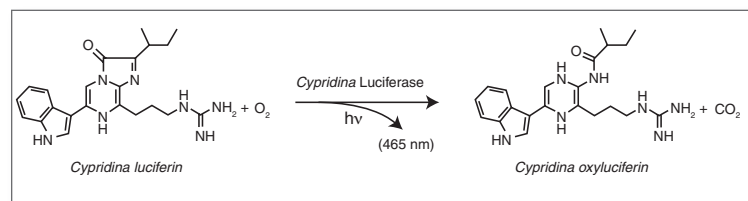
BioLux *Cypridina* Luciferase Substrate is supplied in lyophilized form to ensure long shelf life and maximum stability.

Description:

The BioLux *Cypridina* Luciferase Starter Kit contains a set of *Cypridina* Luciferase-expressing vectors and the reagents necessary for assaying the *Cypridina* Luciferase (CLuc) activity. The pSV40-CLuc Control Plasmid is a mammalian expression vector encoding the secreted *Cypridina* Luciferase from the Ostracod *Cypridina noctiluca* (1,2) under the control of the constitutive SV40 promoter. pSV40-CLuc can be used as a control to determine transfection efficiency. The pCLuc-Basic 2 Vector lacking promoter elements is a cloning vector for mammalian expression. pCLuc-Basic 2 contains a multiple cloning site (MCS) upstream of the CLuc coding sequence. In addition, the neomycin resistance gene in the pCLuc-Basic 2 cloning vector allows selection for stable integration of the plasmid into the mammalian cell genome.

Cypridina Luciferase is a 62 kDa protein with a native signal peptide at the N-terminus allowing it to be secreted from mammalian cells. This luciferase does not require ATP and catalyzes the oxidation of its luciferin substrate in a photochemical reaction (Figure 1). The substrate for *Cypridina* is different than coelenterazine, which is the common substrate of other marine luciferases including *Renilla* and *Gaussia*.

Figure 1: The photochemical reaction catalyzed by *Cypridina* Luciferase.



Properties of *Cypridina* Luciferase:

Cypridina Luciferase is secreted from cells by virtue of its natural signal peptide and its luminescence can be measured from the supernatant of transfected cells. Therefore, cell lysis is not necessary (Figure 2).

Secreted CLuc is a very stable protein. Because of this property, the activity measured from the supernatant reflects the amount of protein accumulated up to the time of sampling. Multiple samples can therefore be obtained from the same transfected cells (Figure 2).

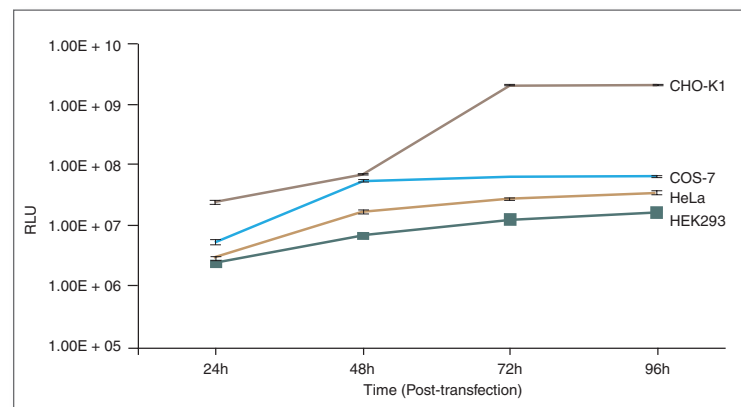
Secreted CLuc is thermally stable at 55°C (Figure 3A), which is the typical inactivation temperature of most viruses.

Secreted CLuc remains active in the presence of β -mercaptoethanol, which is commonly present in the complete culture medium of mouse ES cells (Figure 3B).

The CLuc assay is very sensitive, allowing detection of very small amounts of *Cypridina* Luciferase activity (Figure 4).

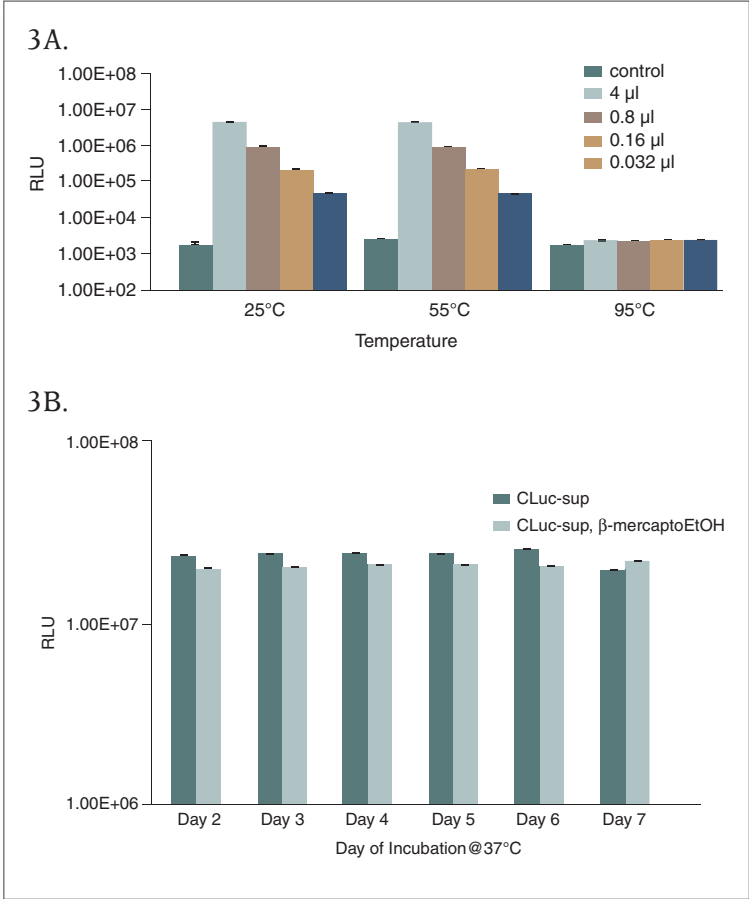
Although the light reaction catalyzed by *Cypridina* has an initial emission half-life of approximately 5 minutes, light production continues to decay slowly, and is readily detectable 25 minutes after substrate addition (Figure 5).

Figure 2: Activity of secreted CLuc at different time points.



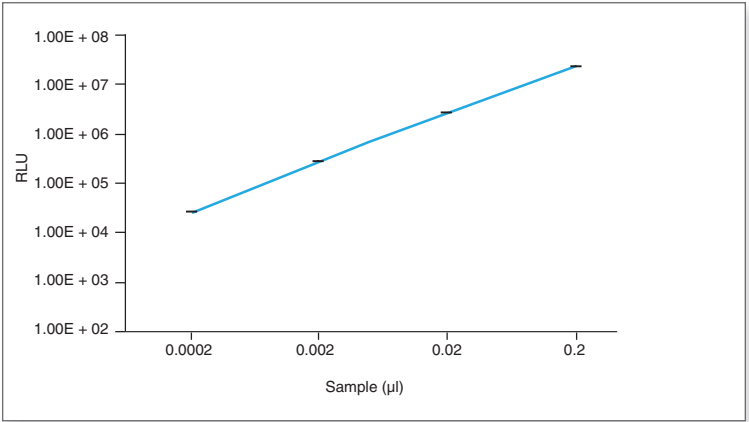
Supernatants of transfected cells were collected and medium was replaced each day for 4 days. These supernatants were stored at -20°C until the last samples were obtained. Relative Light Units, RLU.

Figure 3: Stability of *Cypridina* Luciferase.



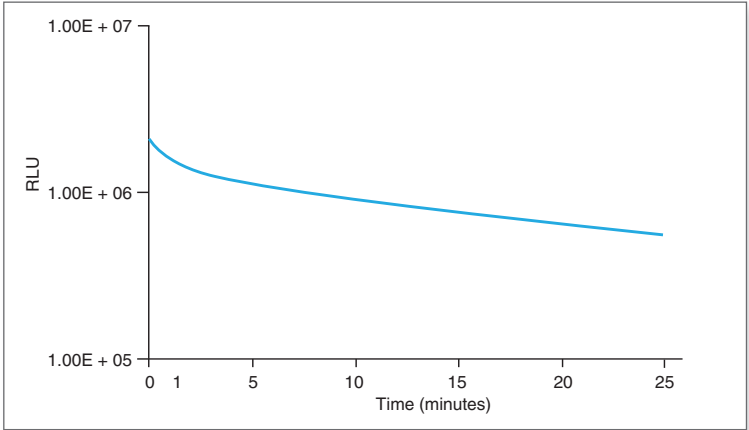
A) Supernatant obtained from stable CLuc-expressing cells was incubated at 55°C or 95°C for 30 minutes and allowed to cool to room temperature (25°C). Five-fold serial dilutions of supernatant were assayed for CLuc activity. The control is the supernatant of the parental cells from which the stable cell line was created. (B) Supernatants from stable CLuc-expressing cells, grown in medium with or without β-mercaptoethanol, were incubated at 37°C and assayed each day for 7 days.

Figure 4: Sensitivity of *Cypridina* Luciferase assay.

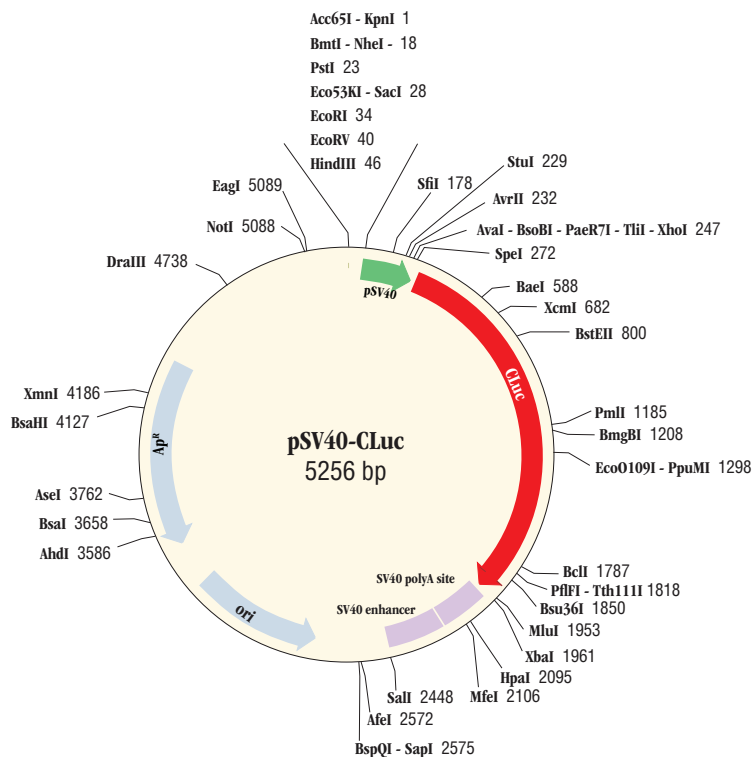


The CLuc activity was assayed from 10-fold serial dilutions of the supernatant from a stable CLuc-expressing cell line.

Figure 5: Kinetics of light emission.



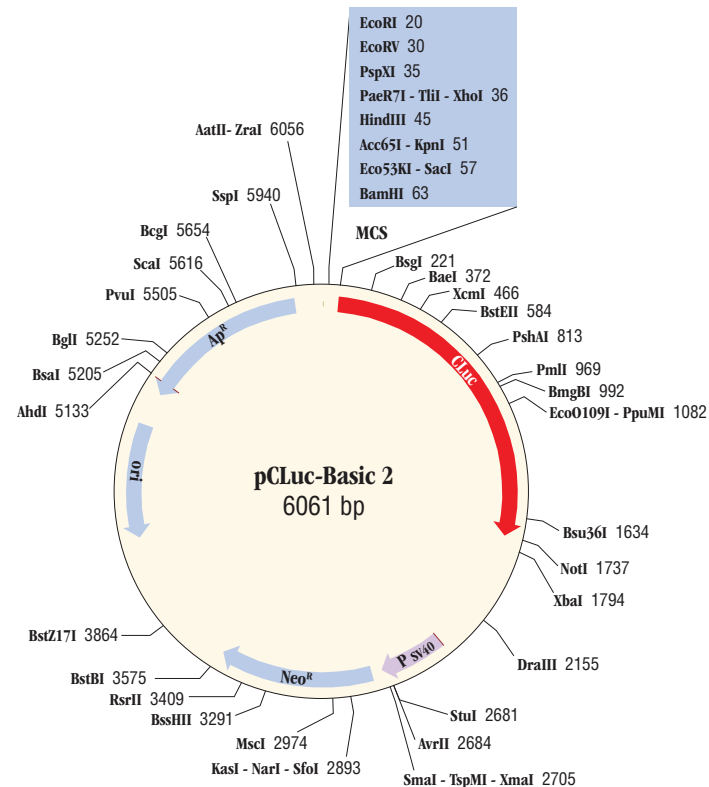
The *Cypridina* Luciferase kinetic assay was performed with the supernatant from HeLa cells transiently transfected with a CLuc-expressing vector. The assay solution was prepared and incubated at room temperature for 30 minutes before use.



1 GGTACCAACCGATCGATCGCTAGCTGCAGAGCTCGAATTGCATATCAAGCTTGCATCTCAATTAGTCAGC 70
SV40 promoter

Features of pSV40-CLuc

- Polylinker MCS: 1–51
- SV40 promoter: 51–246
- CLuc ORF: 291–1952
- Start codon of CLuc: 291–293
- Stop codon of CLuc: 1950–1952
- Signal peptide: 291–344
- SV40 poly-A site: 1967–2188
- SV40 enhancer: 2195–2441
- Bacterial replication ori (pMB1): 3347–2759
- Ampicillin resistance gene: 4378–3518



1 GACGGATCGGGAGATCTTGAATTCTGCAGATATCCTCGAGCCCAAGCTT 50
EcoRI EcoRV PspXI HindIII
XhoI
TliI
PaeR7I
51 GGTACCGAGCTCGGATCGCCACCATGAAGACCTTAATTCTTGCCGTTGC 100
KpnI SacI BamHI
Acc65I M K T L I L A V A...

Features of pCLuc-Basic 2

- Polylinker MCS: 20–68
- Start codon of CLuc: 75–77
- Stop codon of CLuc: 1734–1736
- Signal peptide: 75–128
- Synthetic poly-A site: 1745–1793
- neoR (SV40) promoter: 2379–2714
- Neomycin resistance gene: 2766–3560
- Bacterial replication ori (pMB1): 4894–4306
- Ampicillin resistance gene: 5925–5065

Transfection Guidelines

IMPORTANT:

Use good quality plasmid DNA, i.e., CsCl or standard maxiprep. Do not use mini-prep DNA.

Use proliferating mammalian cultures, i.e., regularly passaged cells.

Use complete growth medium without antibiotics and antimycotics to plate cells for transfection.

Use pSV40-CLuc to establish the transfection efficiency in a particular cell line.

Titrate the amount of plasmid and transfection reagent to achieve optimal transfection efficiency.

Protocol for Creating Stable Cell Lines:

1. Transfect several 60 mM plates with a pCLuc-Basic 2-derived plasmid with an appropriate transfection agent for your cells.
2. Replace the medium, return the cells to the incubator and continue with the incubation for another 24 hours.
3. Start the selection by preparing growth media containing a range of G418 concentrations (e.g. 250 µg/ml, 500 µg/ml, 1 µg/ml etc.).
4. Replace the medium in the petri plates with the G418-containing medium and continue with the incubation for 24 hours.
5. Continue to replace the medium daily until the majority (~99%) of cells are dead, then stop replacing the medium and allow the plate to incubate (for ~7–10 days) until colonies begin to appear.
6. Replace the medium before starting the isolation of colonies.
7. Transfer colonies to a 96-well plate.
8. Replace the medium in the petri plate before returning it to the incubator. Cells can be transferred to 96-well plates until colonies begin to merge on the petri plate.
9. Replace the medium for the isolated clones every 2-3 days until the cell density reaches ~80%.

Note: Use medium containing the concentration of G418 from which the colonies were isolated.
10. Assay the CLuc activity in the supernatants to screen for positive clones.
11. Expand the clonal cultures to the 48-well format.
12. Repeat the expansion (to 24-well, to 12-well, to 6-well plates, to a T25 flask and etc.) until sufficient cell stock has been generated for freezing.

Reconstitution of BioLux *Cypridina* Luciferase Substrate:

IMPORTANT:

Lyophilized substrate can be stored at –20°C for up to 2 years without loss of activity.

Reconstituted substrate should be prepared only if you plan to perform the assays in the near future.

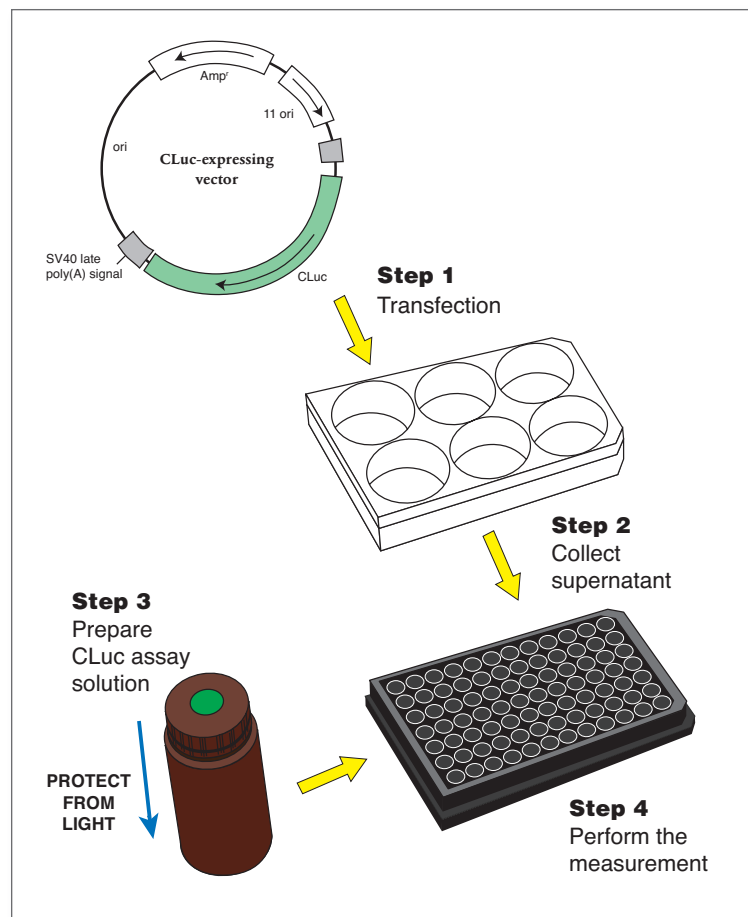
Aliquots of reconstituted substrate can be stored at –80°C for up to 2 months.

Preparation of Reconstituted Substrate (100X solution):

1. Briefly centrifuge the thawed BioLux *Cypridina* Substrate Solvent vial at 12,000 rpm for 30 seconds.
2. Add 0.5 ml of absolute ethanol (not included) to the BioLux *Cypridina* Substrate Solvent and mix well.
3. Carefully lift the rubber cap of the lyophilized BioLux *Cypridina* Substrate vial and add 60 µl of the ethanol-solvent mixture (for small kit) or 600 µl of the ethanol-solvent mixture (for large kit) to the lyophilized BioLux *Cypridina* Luciferase Substrate.
4. Gently mix to dissolve the substrate (do not vortex, do not create bubbles by pipeting) and incubate at 4°C for 10–15 minutes (protect from light).
5. Gently mix again and incubate at 4°C for an additional 10–15 minutes (protect from light).

Note: Be sure to dissolve the residual lyophilized powder on the rubber cap.
6. Aliquot the reconstituted substrate (100X solution) and store at –80°C.

Figure 6: *Cypridina* Luciferase Assays



CLuc Activity Assay Protocols

Protocol I (Luminometers without injectors):

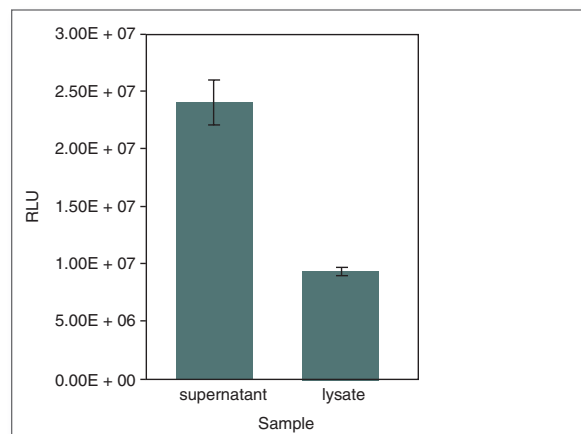
1. Thaw BioLux *Cypridina* Luciferase Assay Buffer (1X) completely to room temperature (protect from light) and mix well before use.
2. Prepare the CLuc assay solution (e.g. for 100 samples add 50 μ l of the reconstituted substrate (100X solution) to 5 ml of BioLux *Cypridina* Luciferase Assay Buffer).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

Protocol II (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 μ l 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 μ l injection, 1–2 seconds of delay and 2–10 seconds of integration.
 6. Pipet samples * (5–20 μ l per well) into a 96-well white (opaque) or black plate.
 7. Prime the injector with the CLuc assay solution and proceed with the measurement.

* Approximately 90% of *Cypridina* Luciferase is secreted out 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 (#B3321), since this lysis buffer is designed to be compatible with *Cypridina*, *Gaussia*, *Renilla*, Firefly Luciferase and β -gal activity assays.

Figure 7: Activity of *Cypridina* Luciferase in the supernatant and lysate from a stable CLuc-expressing cell line.



The CLuc activity was measured from 20 μ l of supernatant (from 500 μ l total volume) and from 20 μ l of cell lysate (100 μ l total lysate volume) of a stable CLuc expressing cell line.

Usage Notes:

Four recommended sequencing primers for pCLuc-Basic 2 Vector are as follows:

pBasic Forward Sequencing Primer (23-mer) (not available at NEB)
5'-GGGGTTCCGCGCACATTCCCG-3' (6020–6042)

pBasic Reverse Primer (25-mer) (not available at NEB)
5'-TCAGAAGCCATAGAGCCACCGCAT-3' (1888–1864)

CLuc 3' End Forward Primer (23-mer) (not available at NEB)
5'-GAGTTCAGAAAGAATGCTACAT-3' (1671–1693)

CLuc 5' End Reverse Primer (24-mer) (not available at NEB)
5'-GTAAGGACAGTCCTGGCAATGAAC-3' (143–120)

Constructs made from the pCLuc-Basic 2 Vector can be used to create stable clonal cell lines using G418 selection.

G418 (Neomycin) should be included in the complete growth media when culturing a stable clonal cell line.

Store the BioLux *Cypridina* Luciferase Assay Buffer (1X) at -20°C for up to 2 years.

The BioLux *Cypridina* Luciferase Assay Buffer (1X), the reconstituted CLuc substrate (100X solution) and the CLuc assay solution must be protected from light.

The linear range of the luminometer must be established. This can be easily done by assaying serial dilutions of a CLuc containing sample (Figure 3), such as the culture medium of CLuc-expressing cells. In addition, the assay solution itself as well as the conditioned medium (culture medium from untransfected cells) should be included to establish the background in the assay.

If excess activity for the instrument range is found, the sample should be diluted in either PBS or 10% serum-containing medium. The integration time can also be reduced, e.g. 2 seconds instead of 5 seconds.

The presence of serum, i.e. culture medium, typically increases the background signal in the assay. For example, the CLuc assay solution alone shows 10^1 – 10^2 RLU; the CLuc assay solution in 10% FBS-containing medium results in 10^2 – 10^4 RLU, depending on the type of medium used.

The recommended integration time for the luminescence measurement is 2–10 seconds. It is important to keep the integration time constant, in order to obtain consistent results.

Frequently Asked Questions:

Can I save the unused portion of the CLuc assay solution?

Ideally the CLuc assay solution is prepared immediately before performing the assays. However, the unused portion of the assay solution can be stored at -20°C for up to 2 days. It must be completely thawed to room temperature (protected from light) and mixed well (do not vortex) before use. Using the previously frozen assay solution will result in approximately a 20% decrease in CLuc activity. The unused portion of the CLuc assay solution should be discarded after one round of freeze-and-thaw.

Does the activity of *Cypridina* Luciferase interfere with that of other luciferases such as *Gaussia*, *Renilla* & *Firefly*?

No. *Cypridina* Luciferase catalyzes the light production of a substrate that is different than those used by *Gaussia*, *Renilla* or *Firefly* luciferases. The secreted CLuc is typically assayed in the supernatant of CLuc-expressing cells; however, the CLuc activity can also be assayed in the cell lysate. If the cell lysate contains *Gaussia*, *Renilla* or *Firefly* luciferase in addition to *Cypridina* Luciferase, one can assay each luciferase independently without cross-reactivity.

What other reporter can I use along with *Cypridina*?

Cypridina can be used in combination with *Gaussia*, *Renilla* and *Firefly* luciferases and *lacZ* reporter in a co-transfection. *Cypridina* and *Gaussia* are both secreted luciferases, producing high bioluminescent signal intensity. Thus, *Cypridina* and *Gaussia* are an ideal reporter pair for co-transfecting mammalian cells (3). For more information on *Gaussia* Luciferase products, please go to the website, (www.neb.com).

References:

1. Nakajima, Y. et al. (2004) *Biosci. Biotechnol. Biochem* 63, 565–570.
2. Yamagishi, K., Enomoto, T. and Ohmiya, Y. (2006) *Anal. Biochemistry*, 354, 15–21.
3. Wu, C., Suzuki-Ogoh, C. and Ohmiya, Y. (2007) *Biotechniques*, 42, 290–292.

Ordering Information

PRODUCT	NEB #	SIZE
BioLux <i>Cypridina</i> Luciferase Starter Kit	E3314S/L	100/1,000 assays
COMPANION PRODUCTS		
pSV40-CLuc Control Plasmid	N0318S	20 µg
pCLuc-Basic 2 Vector	N0317S	20 µg
pCMV-CLuc 2 Control Plasmid	N0321S	20 µg
pTK-CLuc Vector	N0322S	20 µg
pCLuc Mini-TK 2 Vector	N0324S	20 µg
BioLux <i>Cypridina</i> Luciferase Starter Kit	E3314S/L	100/1,000 assays
pSV40-GLuc Control Plasmid	N0323S	20 µg
pGLuc-Basic 2 Vector	N8082S	20 µg
pCMV-GLuc 2 Control Plasmid	N8081S	20 µg
pTK-GLuc Vector	N8084S	20 µg
pGLuc Mini-TK 2 Vector	N8086S	20 µg
BioLux <i>Gaussia</i> Luciferase Assay Kit	E3300S/L	100/1,000 assays
BioLux <i>Gaussia</i> Luciferase Flex Assay Kit	E3308S/L	100/1,000 assays
Anti-GLuc Antibody	E8023S	0.2 ml
Luciferase Cell Lysis Buffer	B3321S	25 ml



USA

New England Biolabs, Inc.
240 County Road
Ipswich, MA 01938-2723
Telephone: (978) 927-5054
Toll Free: (USA Orders) 1-800-632-5227
Toll Free: (USA Tech) 1-800-632-7799
Fax: (978) 921-1350
e-mail: info@neb.com
www.neb.com

CANADA

New England Biolabs, Ltd.
Telephone: (905) 665-4632
Toll Free: 1-800-387-1095
Fax: (905) 665-4635
Fax Toll Free: 1-800-563-3789
e-mail: info.ca@neb.com
www.neb.ca

CHINA, PEOPLE'S REPUBLIC

New England Biolabs (Beijing), Ltd.
Telephone: 010-82378265/82378266
Fax: 010-82378262
e-mail: info@neb-china.com
www.neb-china.com

FRANCE

New England Biolabs France
Free Call: 0800-100-632
Free Fax: 0800-100-610
e-mail: info.fr@neb.com
www.neb-online.fr

GERMANY & AUSTRIA

New England Biolabs GmbH
Telephone: +49/(0)69/305 23140
Free Call: 0800/246 5227 (Germany)
Free Call: 00800/246 52277 (Austria)
Fax: +49/(0)69/305 23149
Free Fax: 0800/246 5229 (Germany)
e-mail: info.de@neb.com
www.neb-online.de

JAPAN

New England Biolabs Japan, Inc.
Telephone: +81 (0)3 5669 6191
Fax: +81 (0)3 5669 6192
e-mail: info@neb-japan.com
www.nebj.jp

SINGAPORE

New England Biolabs Pte. Ltd.
Telephone: +65 6776 0903
Fax: +65 6778 9228
e-mail: sales.sg@neb.com
www.neb.sg

UNITED KINGDOM

New England Biolabs (UK) Ltd.
Telephone: (01462) 420616
Call Free: 0800 318486
Fax: (01462) 421057
Fax Free: 0800 435682
e-mail: info.uk@neb.com
www.neb.uk.com