REPORTER SYSTEMS

# BioLux®
# *Cypridina* Luciferase Starter Kit

Instruction Manual

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

NEB #E3314S
100 assays

## Table of Contents:



## Kit Includes:

| | |
|---|---|
| pSV40-CLuc Control Plasmid | 20 µg |
| pCLuc-Basic 2 Vector | 20 µg |
| BioLux *Cypridina* Luciferase Assay Buffer (1X) | 5 ml |
| BioLux *Cypridina* Luciferase Substrate Solvent† | 0.5 ml |
| BioLux *Cypridina* 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).*

# 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.

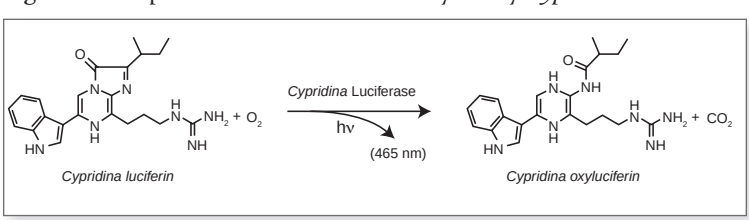
BioLux *Cypridina* Luciferase Substrate is supplied in lyophilized form to ensure long shelf life and maximum stability.

# 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 is typically 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 contains a multiple cloning site (MCS) upstream of the CLuc coding sequence. In addition, the neomycin resistance gene in the pCLuc-Basic 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).**

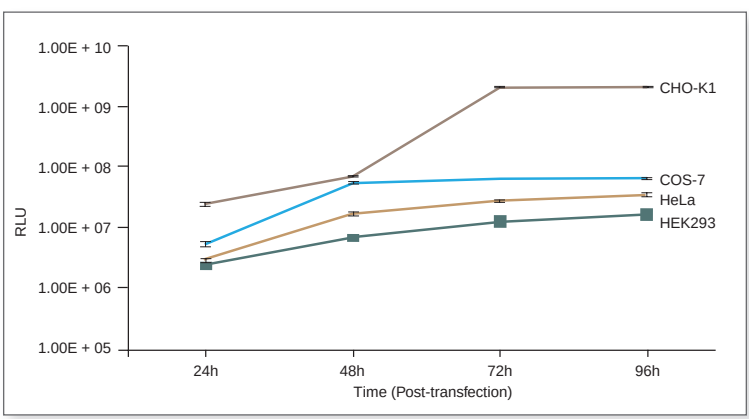
**Secreted CLuc is thermally stable at 55°C (Figure 3A), which is the typical inactivation temperature of most viruses.**

**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).**

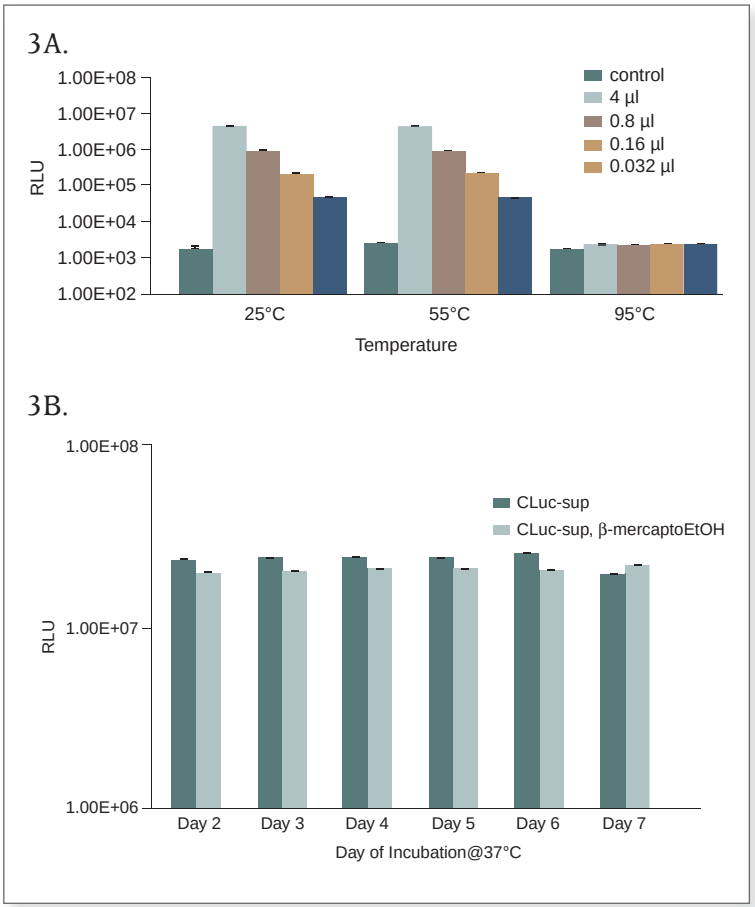
**Although the light reaction catalyzed by Cypridina has an initial emission half-life of approximately 5 minutes, light production continues to decay slowly, and is readily detectable 25 minutes after substrate addition (Figure 5).**

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



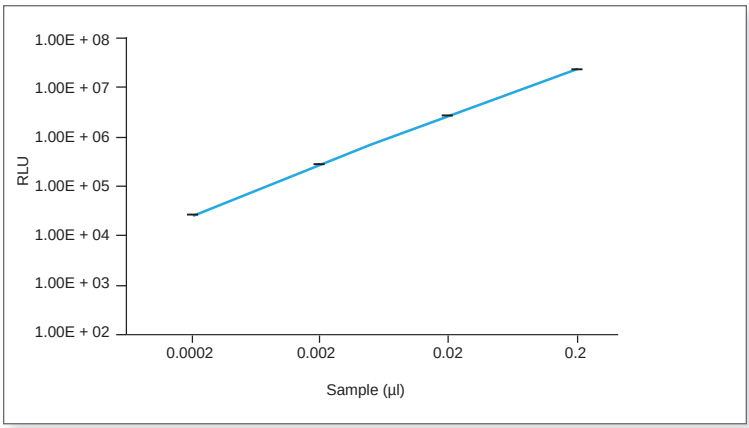

*Supernatants of transfected cells were collected and medium was replaced each day for 4 days. These supernatants were stored at –20°C until the last samples were obtained. Relative Light Units, RLU.*

Figure 3: Stability of *Cypridina* Luciferase.



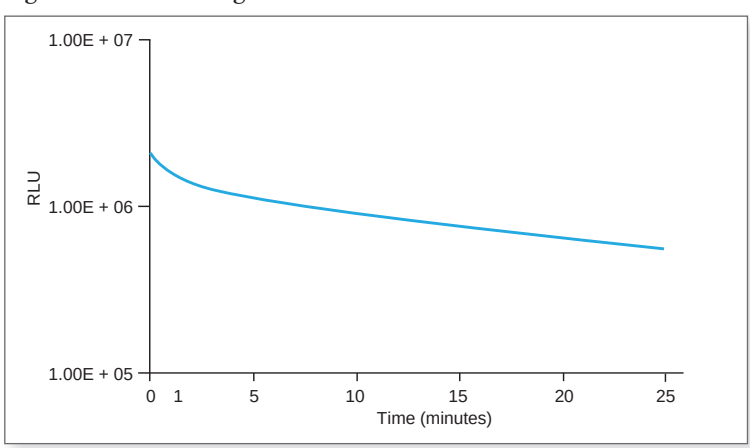

*A) Supernatant obtained from stable CLuc-expressing cells was incubated at 55°C or 95°C for 30 minutes and allowed to cool to room temperature (25°C). Five-fold serial dilutions of supernatant were assayed for CLuc activity. The control is the supernatant of the parental cells from which the stable 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.

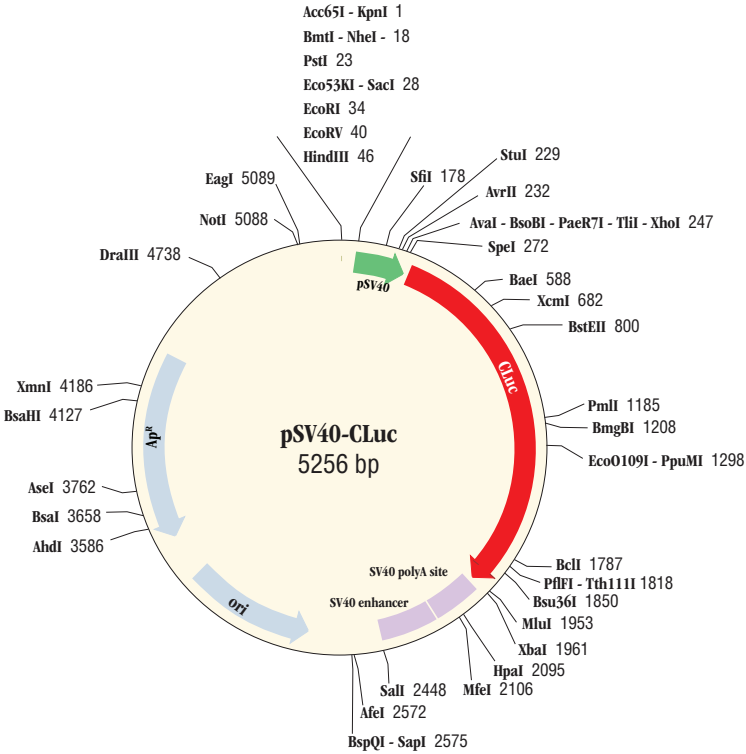



*The CLuc activity was assayed from the 10-fold serial dilutions of the supernatant from a stable CLuc-expressing cell line. The CLuc assay solution was prepared as suggested in the protocol.*

Figure 5: Kinetics of light emission.



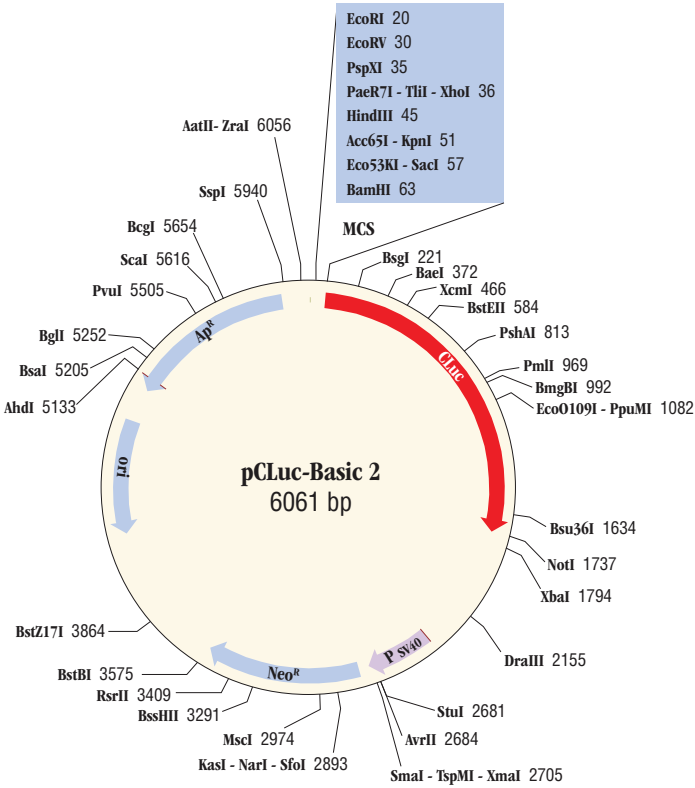

*The assay solution was prepared and incubated at room temperature for 30 minutes before use. The Cypridina Luciferase kinetic assay was performed with the supernatant from HeLa cells transiently transfected with a CLuc-expressing vector.*




```
                          PstI          EcoRI
    KpnI             NheI       Eco53KI          HindIII
    Acc65I           BmtI       SacI         EcoRV
1   GGTACCACCGATCGATCGCTAGCTGCAGAGCTCGAATTCGATATCAAGCTTGCATCTCAATTAGTCAGC  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
- Ampicilin resistance gene: 4378–3518




```
                        EcoRI     EcoRV  PspXI   HindIII
1   GACGGATCGGGAGATCTTGGAATTCTGCAGATATCCTCGAGCCCAAGCTT   50
                                       XhoI
                                       TliI
        Eco53KI                        PaeR7I
    KpnI  SacI  BamHI
51  GGTACCGAGCTCGGATCCGCCACCATGAAGACCTTAATTCTTGCCGTTGC  100
    Acc65I                  M  K  T  L  I  L  A  V  A...
```

**CLuc**

## 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
- Ampicilin resistance gene: 5925–5065

# Transfection Protocols

**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 I (Transient transfection):

1. Plate cells to obtain 70–80% cell density on the day of transfection.
2. Prepare transfection complex mixtures (e.g. 1 µg plasmid and 1–3 µl TransPass D2 (NEB #M2554) in 100 µl serum-free medium per transfection of a 12-well format) (Table 1), and incubate at room temperature for 20–30 minutes before adding to the cells.
3. Return the cells to the incubator and incubate for 24 hours.
4. Harvest some or all of the supernatant (to get rid of floating cells in the supernatant, centrifuge at 900–1500 rpm for 30 seconds) and store at –20˚C until ready to assay the CLuc activity.
5. (Optional) Replace the medium, return the cells to the incubator and continue with the incubation.

   Note: If the experiment requires incubating cells for several days, replacing the media daily is highly recommended. However, some experiments may require allowing luciferase to accumulate over several days. In that case, replacing the medium is not necessary.

Table 1: Plasmid DNA transfection in the presence of serum

| Culture Vessel | Surface ($cm^2$) | Volume of Plating Medium (per well) | DNA in Serum-free Mixture | TransPass D2 in Transfection |
|---|---|---|---|---|
| 96 well | 0.32 | 75 µl | 0.1 µg in 10 µl | 0.1–0.3 µl |
| 48 well | 0.95 | 125 µl | 0.3 µg in 25 µl | 0.3–0.9 µl |
| 24 well | 1.9 | 250 µl | 0.7 µg in 50 µl | 0.7–2.0 µl |
| 12 well | 3.8 | 500 µl | 1.5 µg in 100 µl | 1.5–4.0 µl |
| 6 well | 9.5 | 1 ml | 3 µg in 250 µl | 6–12 µl |
| 60 mm dish | 21 | 2 ml | 6 µg in 500 µl | 12–20 µl |
| 100 mm dish | 55 | 7 ml | 15–20 µg in 1 ml | 34–50 µl |

### Protocol II (Creating stable cell lines):

1. Set up several (4-5) petri plates for transfection and follow Step 1 – Step 4 of Protocol I.
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 the growth media containing a range of G418 concentrations (e.g. 250 µg/ml, 500 µg/ml, 1 µg/ml and 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 together 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 1 year 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.

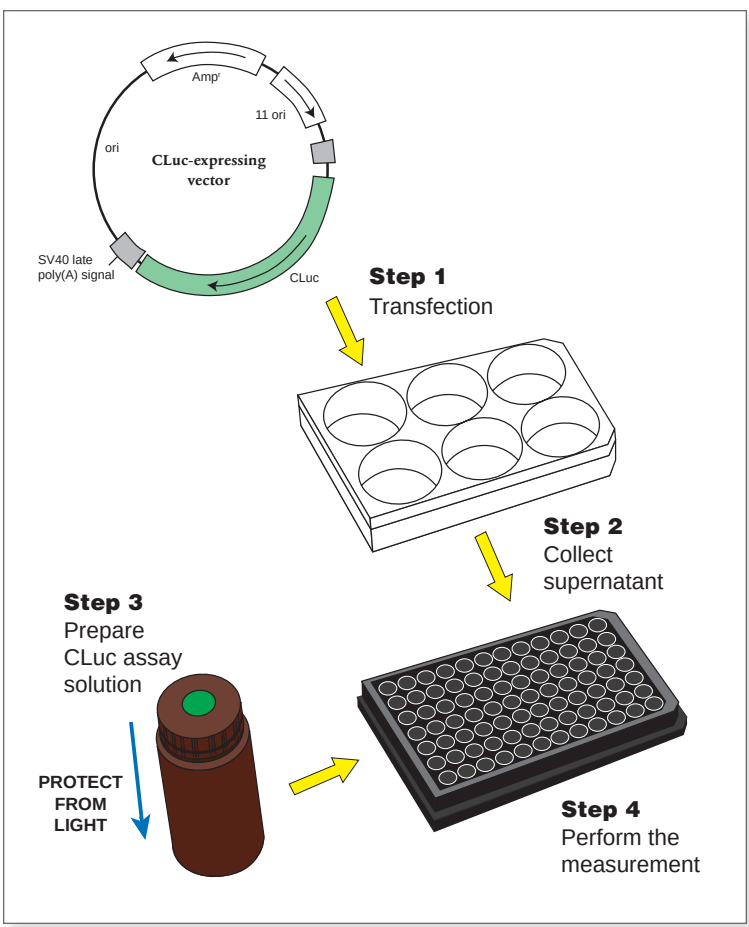
### Preparation of Reconstituted Substrate (100X solution):

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 µl 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 µl per well) into a 96-well white (opaque), black plate or cuvette.
7. Add the CLuc assay solution (50 µl) 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.

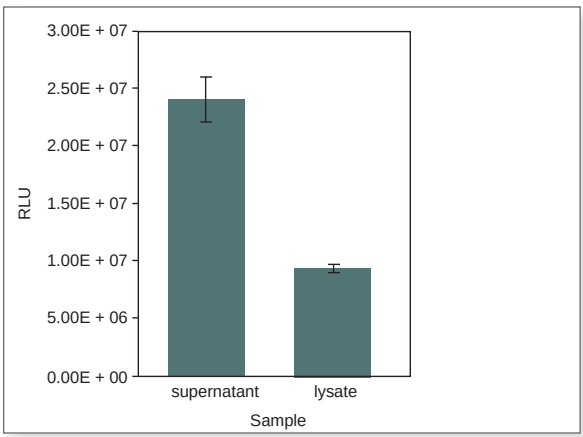
## Protocol II (Injector-equipped luminometers):

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 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.
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 µl of supernatant (from 500 µl total volume) and from 20 µl of cell lysate (100 µl total lysate volume) of a stable CLuc expressing cell line. .*

## Usage Notes:

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

pGLuc-Basic Forwards Sequencing Primer (23-mer) (NEB #S1282)
5´-GGGGTTCCGCGCACATTTCCCCG-3´ (6020–6042)

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

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

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

For improving the transfection efficiency and transfecting difficult-to-transfect cell lines (suspension cells, primary cells, monocytes, etc.), we recommend using TransPass V (NEB #M2561) in combination with TransPass D2 (NEB #M2554) or TransPass D1 (NEB #M2553). For more information on the TransPass Transfection Reagent products, please go to the website (www. neb. com).

Constructs made from the pCLuc-Basic vector can be used to create stable clonal cell lines using G418 selection (refer to Transfection Protocol II).

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 1 year.

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® *Cypridina* 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 |
| BioLux® *Cypridina* Luciferase Assay Kit | E3309S/L | 100/1,000 assays |
| Luciferase Cell Lysis Buffer | B3321S | 0.2 ml |
| pCMV-GLuc Control Plasmid | N8081S | 20 µg |
| pGLuc-Basic Vector | N8082S | 20 µg |
| pTK-GLuc Vector | N8084S | 20 µg |
| pGLuc Mini-TK Vector | N8086S | 20 µg |
| BioLux® *Gaussia* Luciferase Assay Kit | E3300S/L | 100/1,000 assays |
| BioLux® *Gaussia* Luciferase Flex Assay Kit | E3308S/L | 100/1,000 assays |
| Anti-GLuc Antibody | E8023S | 0.2 ml |
| TransPass™ D1 Transfection Reagent | M2553S | 0.5 ml |
| TransPass™ D2 Transfection Reagent | M2554S | 0.5 ml |
| TransPass™ HeLa Transfection Reagent | M2556S | 2 x 0.4 ml |
| TransPass™ COS/293 Transfection Reagent | M2557S | 1.2 ml |
| TransPass™ HUVEC Transfection Reagent | M2558S | 1.8 ml |
| TransPass™ V | M2561S | 0.6 ml |

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U.S. Patent Nos. 7,718,389 and 7,989,621
U.S. Appln. Serial Nos. 12/588,671 and 13/067,565
Japanese Patent Nos. 4,761,150 and 4,484,429
Japanese Appln. Serial No.: 2006-280827; 2007-536587; 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.

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Version 1.0
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