

Luciferase Reporter Assay Kit User Manual

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I. Introduction

Firefly beetle (*Photinus pyralis*) luciferase is one of the most popular reporter molecules used in molecular biology and biochemistry (Gould & Subramani, 1988; Vieites *et al.*, 1994; Gailey *et al.*, 1997). Luciferase can be used to monitor promoter response activity in bacteria, cultured cells, and transgenic plants or animals. By providing faster results, lower costs, and over a 1,000-fold increase in sensitivity, the luciferase assay has largely replaced the standard ^{14}C chloramphenicol acetyltransferase (CAT) assay.

Our **Luciferase Reporter Assay Kit** provides a simple means for detecting luciferase activity in transformed bacteria or transfected eukaryotic cells. The kit includes a firefly luciferase substrate formulation and an optimized cell lysis buffer. This buffer enhances luciferase recovery and activation when used with *in vitro* assays. The unique formulation of this kit provides high sensitivity, constant light output, as well as convenience and consistency when working with multiple samples.

Background

Firefly luciferase has been reliably expressed as a reporter gene from many expression vectors and in a variety of organisms. Its major use has been to characterize gene regulation—primarily transcriptional control—by correlating variations in luciferase activity with the regulation of promoter and enhancer elements. Performed under optimal conditions, the peak height and integrated total light output from a reaction is proportional to the amount of functional luciferase enzyme. This results in a direct relationship between the amount of light emitted from the sample and the transcriptional activity of the regulatory elements.

Firefly luciferase catalyzes the oxidative carboxylation of luciferin, a reaction with the highest efficiency of any known bioluminescence reaction (Seliger & McElroy, 1960). At the optimal reaction pH of 7.8, light emission peaks at 562 nm. This form of light emission yields a very sensitive non-radioactive assay.

The Assay

Firefly luciferase is a 62,000 dalton protein which is active as a monomer and does not require subsequent processing for its activity. However, several factors may affect the sensitivity and success of the assay including pH, temperature, and substrate concentration. To ensure maximum sensitivity, the assay is performed in the presence of excess ATP, luciferin and Mg^{2+} in a buffer that will maintain a pH of 7.8.

For measurement of expressed luciferase activity *in vitro*, luciferase is extracted from transfected cells through cell lysis. A typical firefly luciferase assay is then carried out in an assay cuvette. ATP, Mg^{2+} and buffer are added to the lysate either separately or as a preformulated solution. The luminescent reaction is then triggered by an injection of luciferin, and the emitted light is recorded.

I. Introduction *continued*

When luciferin is added to a sample containing luciferase, there is an immediate light flash that reaches peak intensity at 0.3–0.5 seconds, and then decays rapidly. This rapid exponential decay is caused by the reaction product, oxyluciferin, which inhibits luciferase activity (Lemasters & Hackenbrock, 1977). To overcome this rapid extinction, the Luciferase Reporter Assay Kit includes Coenzyme-A (CoA), which displaces the inhibiting oxyluciferin product substrate from the enzyme, facilitating its turnover (Airth *et al.*, 1958). Inclusion of CoA in the luciferase assay yields a nearly constant light emission rather than the typical flash kinetics (Figure 1), resulting in a more sensitive assay.

By providing CoA, along with ATP, Mg²⁺ and buffer in a preformulated substrate mix, the Luciferase Reporter Assay Kit ensures maximal sensitivity along with an increased ease of handling. The Luciferase Reporter Assay Kit is suitable for use with any standard transfection experiment utilizing firefly luciferase as a reporter.

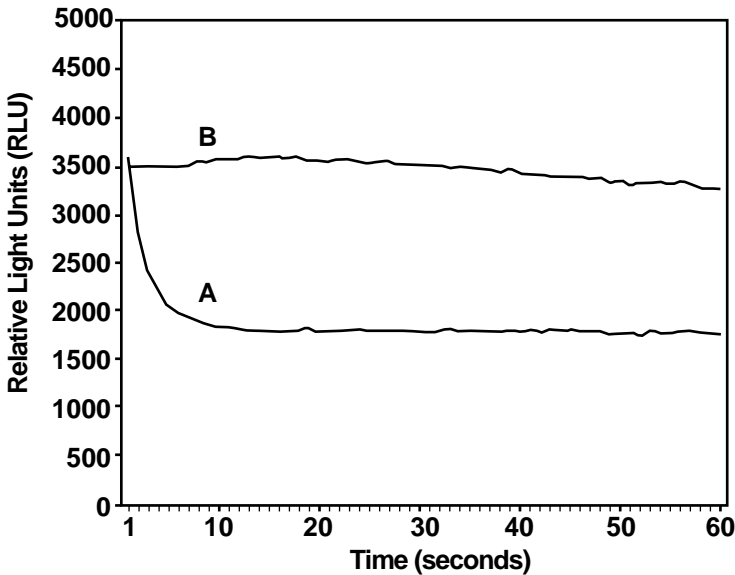


Figure 1. Effect of CoA on firefly luciferase kinetics. Basic luciferase assay (A) and luciferase assay modified by the addition of CoA (B).

II. List of Components

Prior to reconstitution, store all reagents at -20°C .

Substrates A and B must each be reconstituted in 10 ml deionized or distilled water. After reconstitution, Substrates A and B may be stored as aliquots either at -20°C for 6 months or at 4°C for 5 days. Before starting an assay, dilute an aliquot of 3X Cell Lysis Buffer to 1X with deionized or distilled water (see Section V.A).

Reagents provided are sufficient for 100 assays.

- **10 ml Substrate A**
- **10 ml Substrate B**
- **50 ml 3X Cell Lysis Buffer**

III. Additional Materials Required

The following materials are required but not supplied:

- **Phosphate buffered saline (PBS; pH 7.4)**

	<u>Final conc.</u>	<u>To prepare 2 L of solution</u>
Na_2HPO_4	58 mM	16.5 g
NaH_2PO_4	17 mM	4.1 g
NaCl	68 mM	8.0 g

Dissolve the above components in 1.8 L of deionized H_2O . Adjust to pH 7.4 with 0.1 N NaOH. Add deionized H_2O to final volume of 2 L. Store at room temperature.

- **Centrifuge for collecting cells**
- **1.5-ml microcentrifuge tubes**
- **0.5-ml microcentrifuge tubes or 96-well flat-bottom microtiter plate**
Chemiluminescence assays are generally performed in 0.5-ml microcentrifuge tubes. Alternatively, reactions can be performed in white opaque 96-well flat-bottom microtiter plates such as those from Xenopore or Costar.
- **Luminometer (tube or plate), scintillation counter or x-ray film**
- **Lysozyme** for bacterial cell lysis

IV. General Considerations

- Ensure that all reagents have reached room temperature before performing assays.
- Do not repeatedly freeze/thaw sample extracts. Loss of luciferase activity will result.
- We strongly recommend using the Cell Lysis Buffer and protocols supplied with this kit. Sonication or other methods of cell lysis may reduce the sensitivity of the assay.
- Optimization of the Luciferase Assay Kit may be necessary for use with your equipment or samples. Signal detection may become saturated when measuring very high light-emitting samples in a luminometer or scintillation counter. If this occurs, dilute your sample with 1X Lysis Buffer and repeat the assay.
- Chemiluminescent detection of luciferase activity can be performed with a luminometer (tube or plate) or a liquid scintillation counter (LSC). Use of an LSC may result in lowered sensitivity and increased variability between samples due to the need for manual addition of Substrate B (Nguyen *et al.*, 1988). In addition, it is necessary to make specific adjustments to the LSC for the correct detection of the luciferase signal (Fulton & Van Ness, 1993).
- It is also possible to measure luciferase expression via exposure of x-ray film to reactions performed in a white opaque 96-well flat-bottom microtiter plate (Xenopore or Costar).
- Measured levels of luciferase activity are normally stated in relative light units (RLUs), which do not represent an absolute value. If you wish to correlate your relative experimental luciferase activities with an absolute value, you must generate a standard curve for your measuring equipment using purified luciferase. However, it is important to be aware that a purified luciferase standard does not necessarily represent the exact amount of luciferase produced by transfected cells, since the specific activity of the expressed luciferase may differ from the purified luciferase.

V. Assay Procedure

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

Important: Equilibrate all reagents to room temperature before starting the assay.

A. Eukaryotic Cell Lysis

This protocol is optimized for use with eukaryotic cell cultures. For a lysis protocol for bacterial cells, please see Part B below.

1. Prepare an adequate amount of 1X Cell Lysis Buffer by diluting 1 part 3X Cell Lysis Buffer into 2 parts distilled or deionized water (see Table I). The following protocol is designed for use with adherent cultures growing in 35-mm tissue culture plates. If you are using plates, wells, or flasks of a different size, adjust the volume proportionally.
2. Remove media from cell culture plates and rinse twice with phosphate buffered saline (PBS without Ca^{2+} and Mg^{2+} ; see Section III for recipe).
3. Add 200 μl 1X Cell Lysis Buffer to cells and shake at room temperature for 15–20 min. Alternatively, cells may be lysed at 4°C to minimize protease activity. If performing lysis at 4°C, allow cell lysate to reach room temperature before continuing with protocol.
4. Dislodge cells by scraping or pipetting and transfer to a 1.5-ml microcentrifuge tube. Spin cells at 14,000 rpm at room temperature for 1 min to remove cellular debris.
5. Samples should be assayed within 20 min. For measurements that require longer time points or for assays that are to be completed at a later date, extracts may be stored for up to one month at –70°C.

TABLE I. CULTURE PLATE CONVERSION

Plate or Flask Size	Growth Area (cm ²)	Relative Area*	Recommended Volume 1X Cell Lysis Buffer
96 well	0.32	0.04 X	20 μl
24 well	1.88	0.25 X	50 μl
12 well	3.83	0.50 X	100 μl
6 well	9.4	1.20 X	200 μl
35 mm	8.0	1.00 X	200 μl
60 mm	21	2.60 X	500 μl
10 cm	55	7.00 X	1.0 ml
T25	25	3.00 X	500 μl
T75	75	9.00 X	1.2 ml

* Relative area is expressed as a factor of the growth area of a 35-mm culture plate.

V. Assay Procedure *continued*

B. Bacterial Cell Lysis

1. Prepare 1 ml of 1X Cell Lysis Buffer for each 1–10 ml aliquot of bacterial culture by making a 1:3 dilution of 1 part 3X Cell Lysis Buffer to 2 parts distilled or deionized water. Add lysozyme to a final concentration of 1 mg/ml.
2. Centrifuge a 1–10 ml aliquot of bacterial culture. If required, an optimal volume may be determined after initial measurement of activity.
3. Remove and discard supernatant without disturbing cell pellet.
4. Add 1 ml 1X Cell Lysis Buffer and vortex to resuspend cell pellet.
5. Let stand at room temperature for 5–10 min, then centrifuge lysate at 14,000 rpm at room temperature for 1 min to remove insoluble debris.
6. As with eukaryotic samples, assays should be performed within 20 min. For measurements that require longer time points or for assays that are to be completed at a later date, extracts may be stored for up to one month at -70°C .

C. Luciferase Assay

1. Place 20–100 μl cell extract into an assay cuvette. Be sure to use the same volume for each sample.
2. If measurement will be performed on a luminometer or scintillation counter, the recommended measurement time is 10–30 sec (photographic or CCD-type instruments typically require exposures as long as 5 min). Follow the step below that is appropriate for your instrument:
 - a. If your luminometer contains a single automatic injector, manually add 100 μl of Substrate A to the assay cuvette and automatically inject 100 μl of Substrate B within 10 min. Set the delay after the injection and before measurement to 1 or 2 sec.
 - b. If your luminometer contains two automatic injectors, inject 100 μl of Substrate A first, followed by 100 μl of Substrate B. Set the delay between injections to 1 or 2 sec. Set the delay after the second injection and before measurement to 1 or 2 sec.
 - c. If no automatic injectors are used, manually add 100 μl of Substrate A to the assay cuvette. Immediately before measurement, manually add 100 μl of Substrate B to the assay cuvette. The time between adding Substrate B and start of measurement should be as short as possible and consistent from sample to sample.

VI. Troubleshooting Guide

A. Intra-assay Variability

Pipetting error	Use larger sample volumes to minimize variability caused by pipetting error.
Temperature changes	Be sure all reagents have reached room temperature before performing assay.
Allowing sample and buffer to sit for extended periods of time.	Work quickly to minimize the time between adding and initiating the reaction.
Reagent degradation	Store all reagents at -20°C .

B. Abnormally Low Light from Assay

Improper pH	Test pH of each reagent and adjust to 7.8 if necessary.
Improper substrate concentrations	Check that the correct volume of each reagent is being added to the assay reaction and adjust if necessary.
Reagent degradation	Store all reagents at -20°C .
Presence of interfering substances	Be sure to wash cells thoroughly with PBS 2–3 times before performing lysis.

C. High Background

Contaminated reagents	Reagents may become contaminated by carry-over from pipette tips. Be sure to change tips between reaction components and/or samples. Replace component if necessary.
Contaminated injector lines	Flush injector lines thoroughly with distilled water.

VII. References

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VIII. Related Products

For the latest and most complete listing of all
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BD Mercury™ Reporter Systems

- BD Mercury™ Pathway Profiling Luciferase System 1 K2049-1
- BD Mercury™ Pathway Profiling Luciferase System 2 K2052-1
- BD Mercury™ Pathway Profiling Luciferase System 3 K2053-1
- BD Mercury™ Pathway Profiling Luciferase System 4 K2056-1
- BD Mercury™ Pathway Profiling Luciferase System 5 K2057-1

Tet Expression Systems

- BD™ Tet-Off Gene Expression System K1620-1
- BD™ Tet-On Gene Expression System K1621-1
- pBI-L Bidirectional Tet Vector 6151-1

Retroviral Expression Systems

- BD pRevTet-Off™ System K1626-1
- BD pRevTet-On™ System K1627-1
- BD Adeno-X™ Tet-Off Expression System K1651-1
- BD Adeno-X™ Tet-On Expression System K1652-1

BD Creator™ DNA Cloning Kits

- BD Creator™ pDNR-1 Cloning Kit K1670-1

BD Living Colors™ Vectors

- pEGFPLuc Vector 6169-1