Protocol

TD-P Revision 2.1

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Illumination™ Firefly & Renilla Luciferase Enhanced Assay

Procedure for Cotransfection Luciferase Reporter Assay for Purified Luciferase

Introduction

Introduction

Firefly and *Renilla* Luciferase Assays are two of the leading reporter assays in the measurement of gene function and gene regulation both *in vitro* as well as *in vivo*. The luciferase assays are sensitive and convenient due to the absence of endogenous luciferase activity in most cell types and tissue. Firefly luciferase is a monomeric 62 kDa protein typically isolated from the firefly, *Photinus pyralis*, which catalyzes the ATP-dependent D-luciferin in the presence of oxygen and Mg^{2+} to oxyluciferin producing a yellow to greenish light (~560 nm). *Renilla* luciferase is a monomeric 36 kDa protein isolated from the sea pansy *Renilla reniformis* that catalyzes coelenterazine with oxygen to produce a blue light (480 nm).

Quick Start Protocol

This is a general assay overview for quick reference. See the Method on page 4 for complete instructions.

1. Lyse cells in 1X Passive Lysis Buffer (Note: Buffer is ready to use without dilution)
2. Aliquot 20 µl of cell lysate to each reaction tube
3. Prepare firefly and *Renilla* working solutions
4. Add 100 µl firefly working solution to reaction tube and mix by pipetting up and down
5. Immediately read firefly luminescence
6. Add 100 µl *Renilla* working solution **to the same reaction tube** and mix
7. Immediately read *Renilla* luminescence
8. Discard reaction tube and proceed to the next sample

GoldBio's Illumination™ Firefly & *Renilla* Luciferase Enhanced Assay Kit is a combined luciferase assay allowing the sequential measurement of firefly and *Renilla* luciferase activity in the same sample while still retaining the highest degree of sensitivity, reliability and linearity that you want in your assay. Firefly activity is measured first, then the *Renilla* luciferase assay buffer is added to the sample to simultaneously extinguish all firefly luciferase activity to the level of untransformed cells and begins the *Renilla* luciferase activity. This great luciferase combo allows the sequential measurement of both luciferase assays in the same sample without additional hassle. This is a flash-type assay that requires luminescence to be measured immediately after adding the detection reagents to the luciferase sample. Firefly signal decreases 50% and *Renilla* signal decreases 30% after 10 minutes of reaction time (Figure 2), although signal half-life may vary depending on luciferase expression levels.

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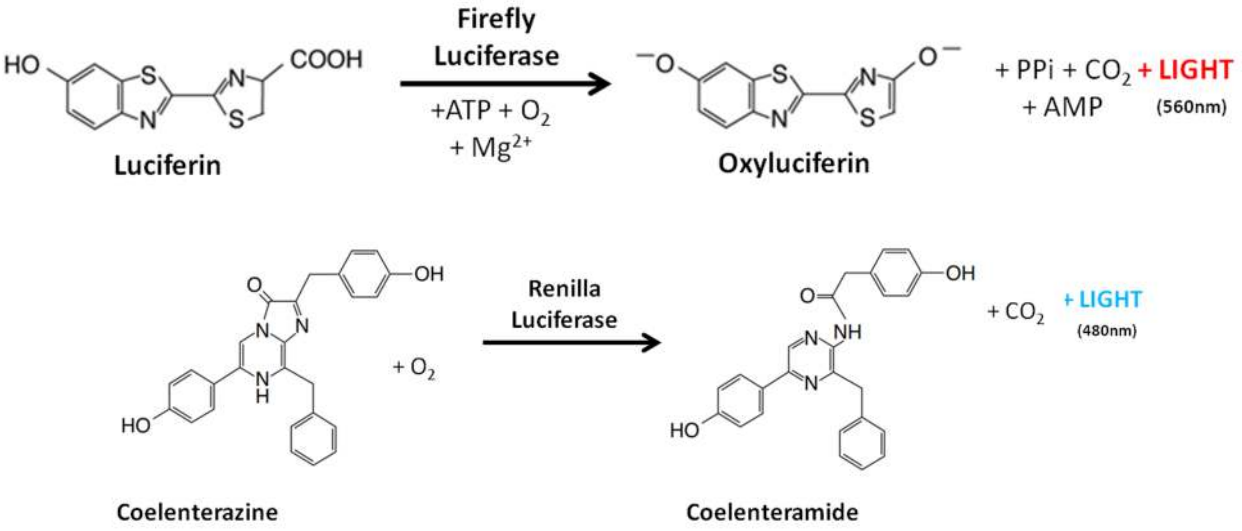
Note: When assaying purified recombinant firefly luciferase in the 1X Passive Lysis Buffer included in the kit, we recommend adding Illumination™ Firefly Luciferase Stabilizer (Catalog # I-940) to the lysis buffer. GoldBio also offers Illumination™ Firefly & *Renilla* Luciferase Enhanced Assay Kit for Purified Luciferase (Catalog # I-921) which includes the stabilizer with the normal components of the regular Illumination™ Firefly & *Renilla* Luciferase Enhanced Assay Kit. The stabilizer is not required for use with cell lysates or purified recombinant *Renilla* luciferase.



Kit Components

Component	I-920-50 (50 assays)	I-920-150 (150 assays)	I-920-1000 (1000 assays)
1X Passive Lysis Buffer	15 ml	15 ml	100 ml
Firefly Luciferase Assay Buffer	5 ml	15 ml	100 ml
Renilla Luciferase Assay Buffer	5 ml	15 ml	100 ml
GoldBio D-Luciferin	1 x 1 mg (LUCK/LUCNA)	3 x 1 mg (LUCK/LUCNA)	2 x 10 mg (LUCK/LUCNA)
GoldBio Enhanced Coelenterazine	1 x 200 µg	3 x 200 µg	1 x 4 mg

Note: Enough lysis buffer is provided to perform the stated number of assays with cells grown in culture plate sizes ranging from 96-well to 24-well plates.

Storage/Handling

Store the kit at -80°C. Firefly and *Renilla* Assay Buffers are stable at -80°C for at least three months from date of receipt. Other components are stable at -20°C or below for at least three months from date of receipt. Kit components are stable for at least 5 freeze/thaw cycles. Product may be shipped on blue ice without reducing performance. Please place at the recommended storage conditions upon receipt of the product.

Assay Design Considerations

Cotransfection experiments

The cotransfection of a control vector together with a reporter vector can suppress expression of the reporter gene. Therefore, preliminary cotransfection experiments should be carried out to determine the optimal ratio of experimental and control plasmids to reliably measure luminescence values above background while minimizing interference in gene expression between vectors. Total mass of transfected DNA also can affect reporter transfection efficiency and/or gene expression, therefore the total mass of DNA used to transfect each sample should be the same. Control DNA (i.e., empty vector) can be used to balance the total amount of DNA per sample.

Recombinant luciferase enzymes

Purified recombinant firefly and *Renilla* luciferase enzymes are available commercially, and can be useful, positive controls for luciferase assays. The 1X Passive Lysis Buffer can be used with recombinant firefly or *Renilla* luciferase without the need to add BSA or other enzyme stabilizer. Note that 1X Passive Lysis Buffer contains protein stabilizers that may affect results if the buffer is used in a protein quantitation assay.

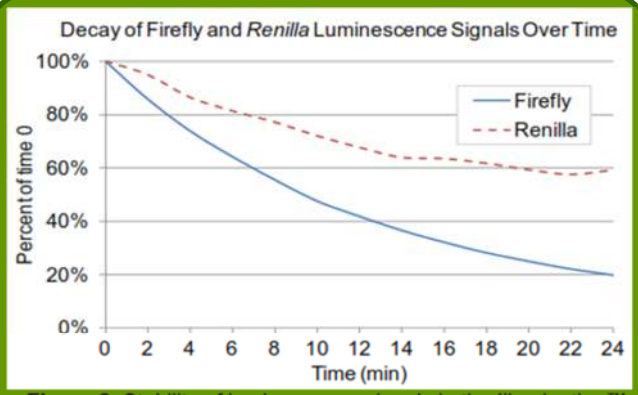


Figure 2. Stability of luminescence signals in the Illumination™ Firefly & *Renilla* Luciferase Enhanced Assay. Firefly or *Renilla measurements were carried out in a white 96-well plate* on cells transfected with firefly luciferase alone or firefly and *Renilla* luciferases. Luminescence was measured using a Bio-Tek H1m microplate reader every 2 minutes for 24 minutes, and RLU values were normalized to the first measurement for each reaction.

Determination of assay background

The expression of a luciferase reporter is quantified by the luminescence produced above background levels. In most cases, background created by the reagent in the absence of luciferase is very low compared to signal with luciferase. However, when measuring low levels of luciferase activity, it is important to subtract the background signal from untransfected cells or cells transfected with a negative control vector from measurements of luciferase activity. The contribution of residual firefly luciferase activity to *Renilla* background can be determined by performing the dual luciferase assay in cells transfected with firefly luciferase alone (without *Renilla* luciferase) to determine

the apparent *Renilla* signal contributed by residual firefly activity. The ratio of firefly and *Renilla* expression levels should be optimized to minimize contribution of residual firefly luminescence to *Renilla* background.

Method

Preparation of Cell Lysates

1. 1X passive lysis buffer is ready to use without dilution and may be stored at 4°C for up to one month.

2. Remove growth medium from cultured cells and gently add a sufficient volume of phosphate buffered saline (PBS) (GoldBio Catalog # P-271) to wash the surface of the culture vessel. Remove the PBS and add 1X passive lysis buffer to each well using the volume recommended below for each type of culture plate:

Wells/plate	Lysis buffer/well
6 well	500 µl
12 well	250 µl
24 well	100 µl
48 well	65 µl
96 well	20 µl

3. Place the culture plates on a rocking platform or orbital shaker with gentle rocking/shaking to ensure complete and even coverage of the cell monolayer with 1X passive lysis buffer. Rock the culture plates at room temperature for 15 minutes.

   Note: Cultures that are overgrown are often more resistant to complete lysis and typically require an increased volume of firefly luciferase lysis buffer and/or an extended treatment period to ensure complete lysis. Lifting cells from the plate will facilitate the process of cell lysis. See GoldBio's Luciferin In Vitro Handbook for more tips and suggestions.

   Note: 1X passive lysis buffer contains protein stabilizers that may affect results of protein quantitation assays.

4. Transfer the lysate to a tube or vial. Place at 4°C until ready to assay. If assay will not be performed on the same day, store lysates at -20°C or -80°C.

   Note (Optional): the lysate can be cleared by centrifugation for 30 seconds at top speed in a refrigerated microcentrifuge and transferred into a new tube.

Preparation of Firefly Working Solution

1. Thaw firefly luciferase assay buffer at room temperature.

2. Prepare 10 mg/ml D-luciferin stock solution. For **I-920-50** or **I-920-150** kits (1 mg D-luciferin), add 100 µl dH_2O to the vial and mix. For **I-920-1000** kit (10 mg D-luciferin), add 1 ml dH_2O to the vial and mix. The stock solution can be stored for at least 6 months at -20°C or below, and is stable for up to 5 freeze/thaw cycles.

3. Prepare enough firefly working solution to perform the desired number of assays (100 µl working solution per assay). Dilute D-luciferin (10 mg/ml) in firefly luciferase assay buffer at a ratio of 1:50. For example, add 20 µl D-luciferin stock solution to 1 ml firefly assay buffer.

> **Note: For best results, working solutions (assay buffer with substrate) should be prepared fresh before each use, and used within 3 hours of preparation. Firefly working solution activity decreases ~10% after 3 hours and ~25% after 5 hours at room temperature.**

Preparation of *Renilla* Working Solution

1. Thaw *Renilla* luciferase assay buffer at room temperature.

2. Prepare 2 mg/ml enhanced coelenterazine stock solution. For **I-920-50** or **I-920-150** kits (200 µg coelenterazine), add 100 µl dH_2O to the vial and mix. For **I-920-1000** kit (4 mg coelenterazine), add 2 ml dH_2O to the vial and mix. The stock solution can be stored for at least 3 months at -20°C or below, and is stable for up to 5 freeze/thaw cycles.

4. Prepare enough *Renilla* working solution to perform the desired number of assays (100 µl working solution per assay). Dilute enhanced coelenterazine (2 mg/ml) in *Renilla* luciferase assay buffer at a ratio of 1:50. For example, add 20 µl enhanced coelenterazine stock solution to 1 ml *Renilla* assay buffer.

> **Note: For best results, working solutions (assay buffer with substrate) should be prepared fresh before each use, and used within 3 hours of preparation. *Renilla* working solution activity is stable for up to 3 hours, but background increases up to 60% after 5 hours at room temperature.**

Firefly & *Renilla* Luciferase Enhanced Assay

The protocol below is for manual assay using a single-tube luminometer. If your luminometer is equipped with automatic injectors, they may be used to dispense one or both working solutions into each luminometer tube or well of a multiwell plate according to the instructions for your instrument.

1. Set up luminometer with parameters recommended for your instrument for dual luciferase assay with an integration time of ~1 second.

2. Add 20 µl of cell lysate into a reaction tube that is compatible with your luminometer.

3. Add 100 µl of firefly working solution to the reaction tube and mix by pipetting up and down several times.

   Note: Do not vortex the tube, which could cause the firefly reaction mix to coat the upper part of the tube and not effectively mix with the *Renilla* working solution in step 5.

4. Immediately place tube in luminometer and record the firefly luminescence measurement.

5. Add 100 µl of *Renilla* working solution to the same reaction tube and mix by pipetting or vortexing.

6. Immediately place tube in luminometer and record the *Renilla* luminescence measurement.

7. Discard the reaction tube, and proceed to the next reaction.

   Note: *Renilla* working solution can be used to measure *Renilla* luciferase activity in the absence of firefly luciferase, but for direct comparison to samples with both firefly and *Renilla* luciferases, you should first add firefly working solution before adding *Renilla* working solution so the final assay volume remains constant between samples. For determination of *Renilla* activity only, firefly working solution can be omitted.

Associated Products

GoldBio Catalog #	Product Name
I-925	Illumination™ Renilla Luciferase Enhanced Assay Kit
I-930	Illumination™ Firefly Luciferase Enhanced Assay Kit
I-940	Illumination™ Firefly Luciferase Stabilizer
I-921	Illumination™ Firefly & Renilla Luciferase Enhanced Assay Kit for Purified Luciferase
LUCK	D-Luciferin, Potassium Salt (Proven and Published™)
LUCNA	D-Luciferin, Sodium Salt (Proven and Published™)
L-710	Dual Luciferase Passive Lysis Buffer