Application Note

# DUAL-LUCIFERASE® REPORTER SYSTEM WITH THE LUMAT TUBE LUMINOMETER


## Abstract

The Dual-Luciferase® Reporter (DLR™) Assay System from Promega is a popular commercial dual reporter gene assay using firefly luciferase as reporter for the promoter of interest and renilla luciferase as internal control reporter. In this application note, the Lumat tube luminometer was validated for its compatibility with the DLR™ Assay System and used to measure samples with a broad range of firefly:renilla ratios. In addition, several mixing procedures were compared. Results show that the Lumat is an excellent solution to measure DLR™ assays and is validated as DLReady™.


## Introduction

Reporter genes have become a very valuable tool in studies of gene expression. They are widely used in biomedical and pharmaceutical research and also in molecular biology and biochemistry.

The main purpose of the reporter gene assay is to investigate the promoter of a gene of interest, i.e., the regulation of its expression. This can be done by linking the promoter of interest to an easily detectable gene, such as the gene for a luciferase, which catalyses a reaction that produces light.

Reporter gene assays based on luminescence are very popular for a variety of reasons:

- They have high sensitivity (typically 10 to 1,000 times higher than methods based on absorbance or fluorescence).
- Most cell types do not have endogenous luciferase activity that could interfere with the assay.
- Luminescence-based assays have a large dynamic range.
- They are quick to perform.
- Their costs are relatively low.

In order to minimize experimental variability caused by random factors (such as differences in cell number, cell viability or transfection efficiency), dual reporter systems can be used. In such systems, two different luciferase reporter enzymes are expressed simultaneously: one is controlled by the promoter of interest and the other one is controlled by a promoter that gives a stable expression and does not change with the experimental conditions, which is used as internal control for normalization [1].

The Dual-Luciferase® Reporter (DLR™) Assay System from Promega is a popular commercial assay using firefly luciferase as reporter for the promoter of interest and renilla luciferase as internal control reporter. In a first step, a reagent containing the substrate of firefly luciferase (LAR II reagent) is dispensed, and the firefly luminescence


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is measured; in a second step a reagent is dispensed, which quenches the firefly luminescence and starts the renilla luminescence (Stop & Glo® reagent), and then the renilla luminescence is measured [1].

One critical point for the performance of the assay is the quenching of the signal of the firefly luciferase: if there is residual luminescence from the firefly luciferase when the reading of the renilla luciferase is taken, the signal of the internal control will be overestimated, and this could lead to wrong results.

In this application note, the Lumat is tested in several ways:

1. Following the validation procedure from Promega for the DLR™ Assay System.
2. Measuring samples with a broad range of firefly:renilla ratios using the DLR™ assay system.
3. Comparing 3 different mixing methods: pipette, vortex and injector.

## Materials

- Lumat Reporter Gene from Berthold Technologies (Id. Nr. 71450-20).
- Renilla luciferase, 0.78 mg/mL, from Promega (Part # E359).
- Firefly luciferase - QuantiLum® Recombinant Luciferase, 12.4 mg/mL, from Promega (Cat# E1701).
- Bovine Serum Albumin, Acetylated (BSA), 10 mg/mL, from Promega (Cat# R3961).
- Dual-Luciferase® Reporter Assay System from Promega (Cat# E1910).
- 5 mL luminescence tubes, 12 x 75 mm, from Berthold Technologies (Id. Nr. 09778).
- Nuclease-Free Water from Promega (Cat# P1193).
- Vortex
- Pipettes and pipette tips (various volumes).

## Methods

Reagents were prepared according to the manufacturer's instructions. To prepare the luciferase dilutions, a dilution buffer was prepared by mixing 1 mL of Passive Lysis buffer from the DLR™ kit with 0.5 mL of BSA 10 mg/mL and 3.5 mL of nuclease-free water.

In order for a luminometer to be validated for the DLR™ Assay System, the instrument has to pass 3 different tests:

1. Tubing adsorption: this test shows whether the tubing used in the instrument injectors has an effect on the DLR assay over time. 20 µL of 50:1 firefly:renilla solution are measured in 12 replicates. The test passes if signal after 10 minutes with reagents standing in the tubing is ≥95% of the signal before incubation.
2. Firefly luciferase quenching: this test shows if the injection system provides enough mixing for signal of firefly luciferase to be totally quenched. 20 µL of firefly luciferase 3.05 x $10^{-5}$ µg/µL are measured in 24 replicates. As there is no renilla luciferase in this solution, the signal of the 2$^{nd}$ measurement is unquenched firefly signal. The test passes if firefly signal after dispensing the Stop & Glo® reagent is quenched at least 10,000 times.
3. Consistency: this test shows if results are consistent in 24 replicates of 20 µL with 2 different firefly:renilla ratios (50:1 firefly:renilla and 50:1 renilla:firefly). The test passes if CV of the measurement is ≤5% both for firefly and renilla signals.

To assess the linearity of the assay, a dilution series of firefly luciferase with concentrations ranging from $10^{-19}$ up to $10^{-15}$ mol/µL was prepared and mixed 1:1 with a solution of renilla luciferase $10^{-19}$ mol/µL; this gives firefly:renilla ratios from 1:1 to 10,000:1. 20 µL of this mix were transferred to luminescence tubes in triplicate. The final quantity of renilla luciferase per tube was $10^{-18}$ mol in all tubes, and the quantity of firefly luciferase was $10^{-18}$, $10^{-17}$, $10^{-16}$, $10^{-15}$ and $10^{-14}$ mol/tube. Blank tubes containing dilution buffer only were also measured in triplicate. The firefly signal was divided by the renilla signal to calculate the luciferase ratio.

To perform the measurement, basic procedure was:

1. Dispense 100 µL of LAR II
2. 2 s delay
3. Luminescence measurement for 10 s (firefly signal)
4. Dispense 100 µL of Stop&Glo®
5. 2 s delay
6. Luminescence measurement for 10 s (renilla signal)

To assess the effect of different mixing methods on the performance of the assay, 3 series of tube were prepared and measured using different dispensing and mixing methods:

1. Vortex: reagents were dispensed using a manual pipette followed by mixing using a vortex during the delay of 2 seconds.
2. Injector: reagents were dispensed using the automatic injectors of the Lumat. Mixing is provided by the pressure of the jet of liquid.
3. Pipette: reagents were dispensed using a manual pipette followed by mixing by pipetting up and down 5 times inside the tube.

## Results

Results of all 3 tests of the validation are summarized in **Table 1**.

Test 1 (adsorption test) passes if signal after 10 minutes with reagents standing in the tubing is ≥95% of the signal at time = 0. Results obtained with the Lumat were 99.4% for the firefly measurements and 98.4% for the renilla measurements, indicating virtually no tubing adsorption.

Test 2 (quenching test) passes if quenching is at least 10,000. Average quenching obtained with the Lumat was above 45,000 (table 1), exceeding the required quenching by more than 4 times. Looking at the values of individual wells, the lowest quenching obtained was 21,003, also exceeding the required value of 10,000.

Test 3 (consistency test) passes if CV of the measurement is ≤5%. CV of the measurements performed with the Lumat was ≤4.6% in all cases.

| **Test 1** | | | **Test 3** | | |
|---|---|---|---|---|---|
| | Firefly | Renilla | 50:1 Firefly:Renilla | Firefly | Renilla |
| Average t=0 | 80,854,536 | 8,544,254 | Average | 70,307,603 | 7,995,953 |
| Average t=10 min | 80,347,922 | 8,405,207 | Std. Dev | 3,156,878 | 367,559 |
| Activity (%) | **99,4** | **98.4** | CV (%) | **4.49** | **4.60** |
| **Test 2** | | | 50:1 Renilla:Firefly | Firefly | Renilla |
| | Firefly | Renilla | Average | 1,139,225 | 616,182,994 |
| Average | 46,915,919 | 1,040 | Std. Dev | 30,173 | 14,594,554 |
| Quenching | | **45,104** | CV (%) | **2.65** | **2.37** |

***Table 1:*** Results of tests 1, 2 and 3. Number of replicates: 12 in test 1, 24 in tests 2 and 3. Results in RLU unless otherwise indicated. Quenching is the average of Firefly/Renilla ratio of individual wells.

The firefly/renilla ratios show excellent linearity in the whole concentration range tested, with a correlation coefficient $R^2$ of 0.9999 (Fig. 1).

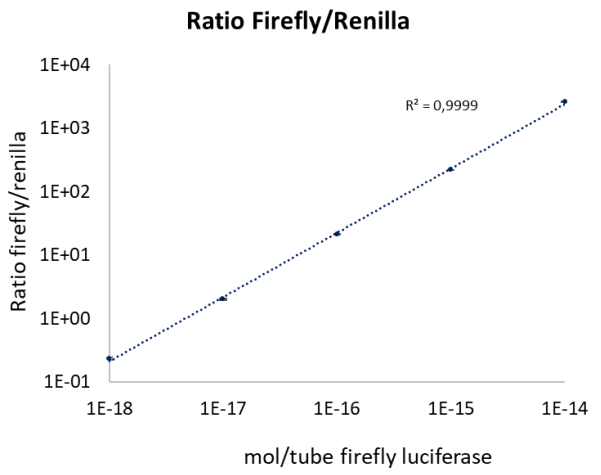


**Figure 1.** Firefly/renilla ratios of the different mixes. Error bars represent standard error of the mean. All data points measured in triplicate.

To compare the effect of the different mixing methods, the ratio between the renilla signal in the tubes with the highest firefly concentration (10,000:1 firefly:renilla) and the tubes with the lowest firefly concentration (1:1 firefly:renilla) was calculated: if the firefly signal is totally quenched, the ratio should be close to 1, indicating no contribution of unquenched firefly activity to renilla signal. Results show that pipetting up and down 5 times does not provide enough mixing to totally quench the firefly signal: while the signal of renilla in tubes mixed either with the injectors or with the vortex is virtually 1, the ratio is higher than 2 in the tubes mixed pipetting up and down, indicating that the signal coming from unquenched activity of firefly luciferase is even higher than the signal coming from renilla luciferase. In fact, using this mixing method, some residual firefly activity is already visible at firefly:renilla ratios of 100:1 (data not shown). In addition, variability of the renilla signal is very high (as is evident from the high standard error values), indicating inconsistent quenching.

Both vortex and injectors provide excellent mixing, but the coefficient of variation (CV) of the firefly/renilla ratios is lower in the tubes dispensed using the automated injectors of the Lumat than in tubes dispensed manually (3.7% vs 4.9% across all tubes).

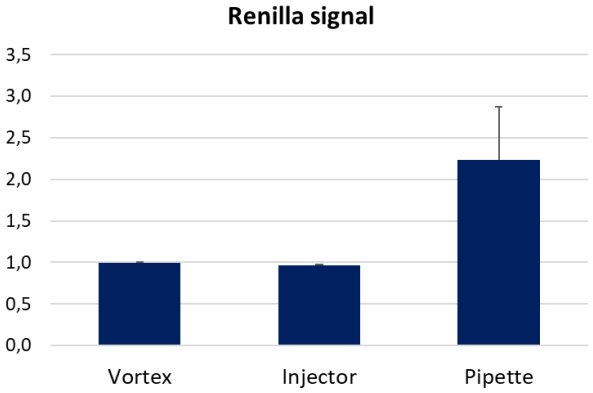


**Figure 2.** Ratio between the renilla signal in the tubes with the highest firefly concentration (10,000:1 firefly:renilla) and the tubes with the lowest firefly concentration (1:1 firefly:renilla). Error bars represent the standard error of the mean. All data points measured in triplicate.

## Discussion and conclusions

The Dual-Luciferase® Reporter Assay is a very popular tool for the study of the regulation of gene expression and, using the right mixing method, the measurement of the second signal (renilla luciferase) does not show any interference from the first signal (firefly luciferase), even at firefly:renilla ratios so high as 10,000:1.

Among the 3 mixing methods tested, the built-in reagent injectors of the Lumat provide the best results: they provide excellent mixing, which ensures that the firefly activity is totally quenched before measuring the renilla signal and, in addition, they have the best reproducibility. If no reagent injectors are available, mixing the tube with a vortex for 2 seconds does also totally quench the firefly signal. On the other hand, mixing by pipetting up and down 5 times produces inconsistent mixing that could be insufficient at high firefly:renilla ratios, and must be avoided.

The Lumat tube luminometer is a flexible and reliable instrument, and the results presented here demonstrate that it is an excellent choice for measuring the Dual-Luciferase® (DLR™) Assay System. It not only passes all validation tests and is listed as DLReady™ [2] but also delivers outstanding linearity across the tested concentration ranges and firefly:renilla ratios. This makes it a highly effective solution for investigating gene expression regulation using Promega's Dual-Luciferase® Reporter Assay System.

## References


1. Sherf, B.A., Navarro, S.L., Hannah, R.R., Wood, K.V. (1996). Dual-Luciferase™ Reporter Assay: An Advanced Co-Reporter Technology Integrating Firefly and Renilla Luciferase Assays. Promega Notes Magazine 57, 2-9.
2. DLReady™ Validated luminometers: https://www.promega.com/en/resources/guides/lab-equipment-and-supplies/dlready-validated-luminometers/


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