

APPLICATION NOTE

Highly sensitive dual luciferase detection with the SpectraMax DuoLuc Reporter Assay Kit

Introduction

Firefly luciferase is a widely used reporter to study gene regulation and function. It is a very sensitive reporter due to the lack of any endogenous luciferase activity in mammalian cells or tissue. Firefly luciferase is a 62-kDa protein that is active as a monomer and does not require subsequent processing for its activity. It catalyzes ATP-dependent oxidation of D-luciferin with the resulting emission of light (Figure 1A). Luciferase from the sea pansy *Renilla reniformis* is often used in multiplexed luciferase assays as a second reporter for normalizing transfection efficiency and for studying gene regulation and function. *Renilla* luciferase catalyzes coelenterazine oxidation by oxygen to produce light (Figure 1B).

The SpectraMax® DuoLuc™ Reporter Assay Kit enables highly sensitive quantitation of both firefly and *Renilla* luciferases in mammalian cells. Serial injection of two optimized detection reagents allows the luciferases to be assayed in the same microplate well. Automated addition of firefly working solution by injection initiates the firefly luciferase luminescence reaction, and subsequent addition of *Renilla* working solution via a second injection quenches the firefly luminescence and adds Aquaphile™ coelenterazine to initiate the *Renilla* reaction. The result is a two-signal assay system that enables normalization of experimental signal to a control. The assay is ideal for use with the SpectraMax® i3x Multi-Mode Microplate Reader with Injector Cartridge and SmartInject™.

Materials

- SpectraMax DuoLuc Reporter Assay Kit (Molecular Devices cat. #R8361)
- HeLa cells (ATCC cat. #CCL-2)
- pGL4.13[*luc2*/SV40] firefly luciferase expression vector (Promega cat. #E668A)
- pGL4.75[*hRluc*/CMV] *Renilla* luciferase expression vector (Promega cat. #E693A)
- ViaFect™ Transfection Reagent (Promega cat. #E4981)
- Opti-MEM Reduced Serum Medium (ThermoFisher Scientific cat. #31985062)
- SpectraMax i3x Multi-Mode Microplate Reader
 - SpectraMax Injector Cartridge with SmartInject

Benefits

- Detect firefly and *Renilla* luciferase expression in ten cells or fewer per well
- Improve accuracy of results by normalizing reporter expression to a control
- Speed time to results with a preconfigured SoftMax Pro protocol for rapid data analysis

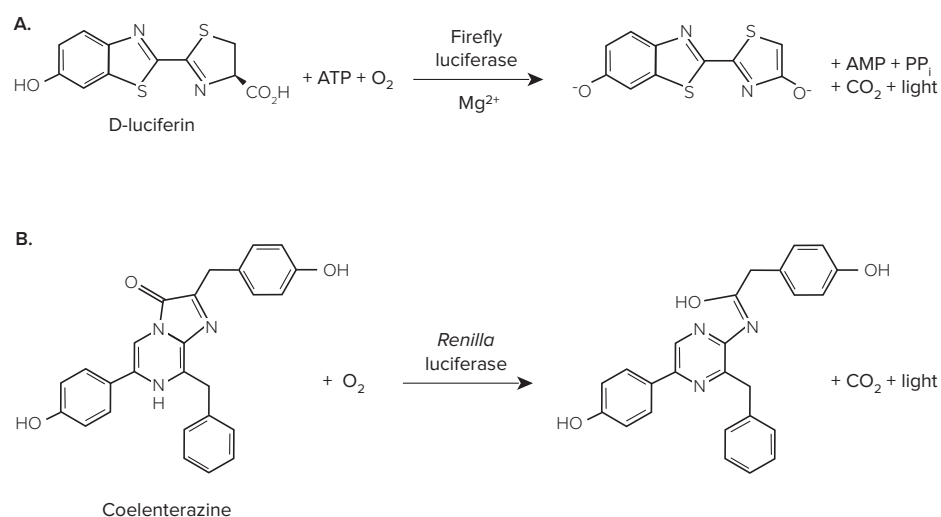


Figure 1. Bioluminescent reactions catalyzed by (A) firefly luciferase and (B) *Renilla* luciferase.

Methods

Cell transfection

HeLa cells were seeded at 2×10^5 cells per well in 6-well tissue-culture treated plates and incubated at $37^\circ\text{C}/5\% \text{CO}_2$ for 24 hours prior to transfection. The pGL4.13[*luc2/SV40*] firefly luciferase expression vector was diluted in Opti-MEM medium to $1 \mu\text{g}/\mu\text{L}$, and the pGL4.75[*hRluc*/CMV] *Renilla* luciferase expression vector was diluted to $100 \text{ ng}/\mu\text{L}$. Three tubes were set up as follows and mixed gently: $400 \mu\text{L}$ of Opti-MEM medium + $2 \mu\text{L}$ ($2 \mu\text{g}$) of pGL4.13[*luc2/SV40*] firefly luciferase expression vector + $2 \mu\text{L}$ (2 ng) of pGL4.75[*hRluc*/CMV] *Renilla* luciferase expression vector. To each tube, $6 \mu\text{L}$ of ViaFect reagent was added, and the contents were gently mixed by tapping. Tubes were incubated for 10 minutes at room temperature to allow complexes to develop. $200 \mu\text{L}$ of transfection complex was added dropwise to each well of the 6-well plate, with gentle swirling to mix.

Cells were incubated at $37^\circ\text{C}/5\% \text{CO}_2$ for 48 hours after transfection and then harvested for assay.

Preparation of cell lysates

Transfected cells in the 6-well plate were trypsinized, divided into ten aliquots, pelleted at 1500 rpm for 5 minutes, and washed once with PBS. PBS was removed, and cell pellets were stored at -80°C until the time of assay.

In preparation for assay, Passive Lysis Buffer and cell pellets were warmed to room temperature, and each cell pellet was lysed in $150 \mu\text{L}$ of Passive Lysis Buffer. Cell lysis was allowed to proceed at room temperature for 15 minutes. Lysate was then serially diluted 1:2 in Passive Lysis Buffer to perform a standard curve spanning concentrations equivalent to 12 to 8700 cells per well. $20 \mu\text{L}$ of each concentration of cell lysate was pipetted into triplicate wells of a solid white 96-well plate.

Luciferase assay setup

All kit components were thawed to room temperature. Firefly Substrate was reconstituted by adding $220 \mu\text{L}$ of water to one vial containing 2.2 mg of lyophilized substrate. Aquaphile coelenterazine was reconstituted by adding $220 \mu\text{L}$ of water to one vial containing $440 \mu\text{g}$ of lyophilized substrate. Note: reconstitution volumes given are for the Explorer Kit. Please refer to the product insert for other kit sizes.

Firefly working solution was prepared by diluting firefly substrate 1:50 in Firefly Assay Buffer. *Renilla* working solution was prepared by diluting Aquaphile coelenterazine 1:50 in *Renilla* Assay Buffer. For one 96-well plate, 11 mL of each working solution was made by adding $220 \mu\text{L}$ of its respective substrate.

A preconfigured SpectraMax DuoLuc assay protocol in SoftMax® Pro Software was used to run the assay and analyze the results with the following parameters:

1. Use injector 1 to add $100 \mu\text{L}$ of firefly working solution to the well.
2. Delay for 2 seconds to allow the reaction to develop.
3. Measure firefly luminescence with an integration time of 5 seconds.
4. Use injector 2 to add $100 \mu\text{L}$ of *Renilla* working solution to the well.
5. Delay for 2 seconds to allow the reaction to develop.
6. Measure *Renilla* luminescence with an integration time of 5 seconds.
7. For each assay well, normalize the RLU value of the first measurement (firefly luciferase) to that of the second (*Renilla* luciferase).

Performance of the SpectraMax DuoLuc Reporter Assay Kit was compared to a competitor's dual luciferase reporter assay.

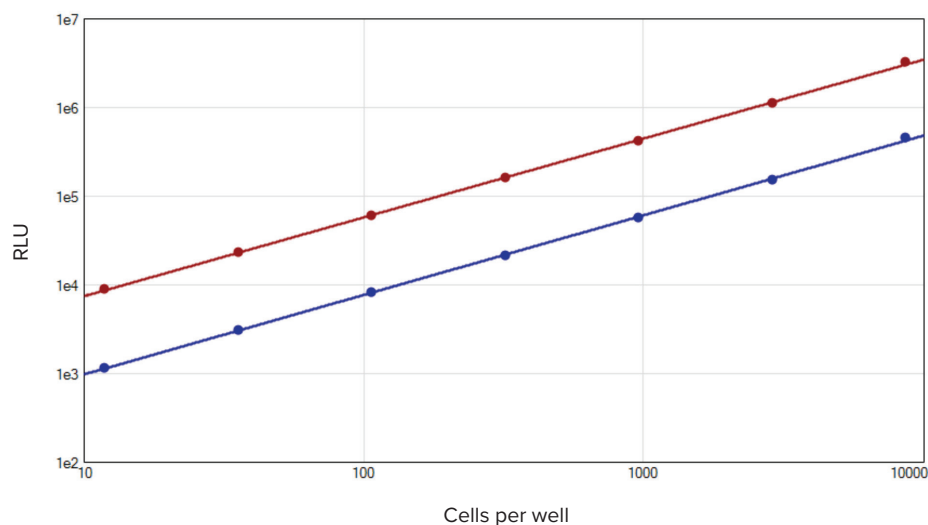


Figure 2. Cell-based assay standard curve. Firefly (red plot) and *Renilla* (blue plot) standard curves were plotted using a log-log curve fit in SoftMax Pro Software ($r^2 = 1.0$ for each). $N=3$ replicates at each cell number.

Assay performance

Relative light units produced by cellular samples using the SpectraMax DuoLuc Reporter Assay Kit were linearly proportional to the sample concentration over the full range tested, with r^2 values of 1.0 for the resulting standard curves (Figure 2). CV values were below 4% for all sets of replicates ($n=3$). The benefit of highly linear data and low %CV values are that one can accurately detect differences in luciferase expression across a wide range of cellular responses. This enables the identification of even small responses to experimental cell conditions leading to scientific discovery for signal transduction, protein-protein interaction, and many other fields.

Standard curves obtained using the SpectraMax DuoLuc Reporter Assay Kit are comparable to those of a competitor's dual luciferase reporter assay (Figure 3). Performance criteria required of instruments used to run dual luciferase assays, including low %CV values, non-absorbance of reagents to the injector tubing, and adequate quenching of firefly signal upon addition of the *Renilla* substrate, are fulfilled by the SpectraMax i3x with Injector Cartridge using the SpectraMax DuoLuc Reporter Assay Kit (data not shown).

SoftMax Pro Software offers the benefit of a preconfigured assay protocol that calculates normalized luciferase values for each well by dividing the firefly luminescence by the *Renilla* luminescence. The results are displayed in a group table and can be easily graphed for results sharing and publication. Normalized values for cell lysates tested here are shown in Figure 4. These were consistent across the range of concentrations assayed here, demonstrating that even if the number of cells in the wells is variable, assay results will be consistent.

Conclusion

The SpectraMax DuoLuc Reporter Assay has been performance optimized on the SpectraMax i3x reader with Injector Cartridge. With a preconfigured protocol to simplify data acquisition and analysis, SoftMax Pro Software completes this total solution for fast, sensitive, and reliable dual luciferase reporter assay results.

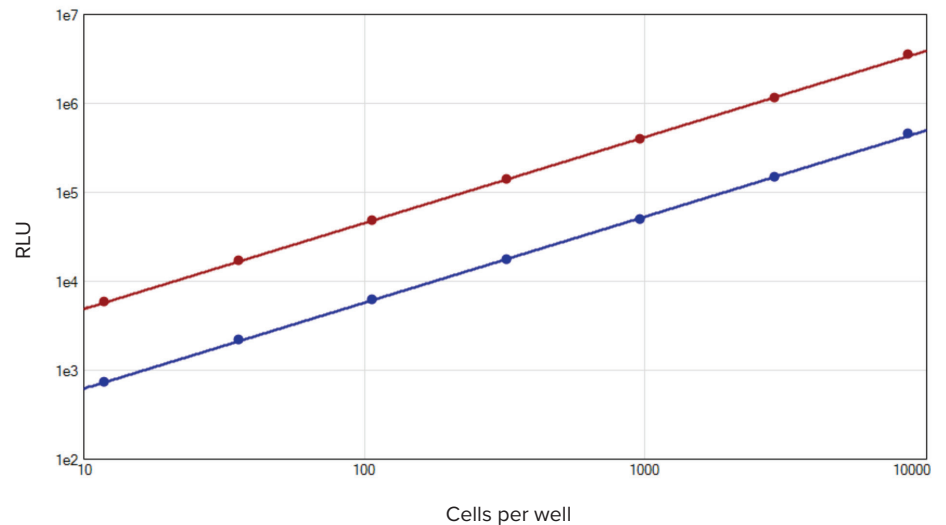


Figure 3. Data from a competitor's dual luciferase reporter assay. RFU values and linearity are comparable to those demonstrated for the SpectraMax DuoLuc Reporter Assay Kit. $N=3$ replicates at each cell number.

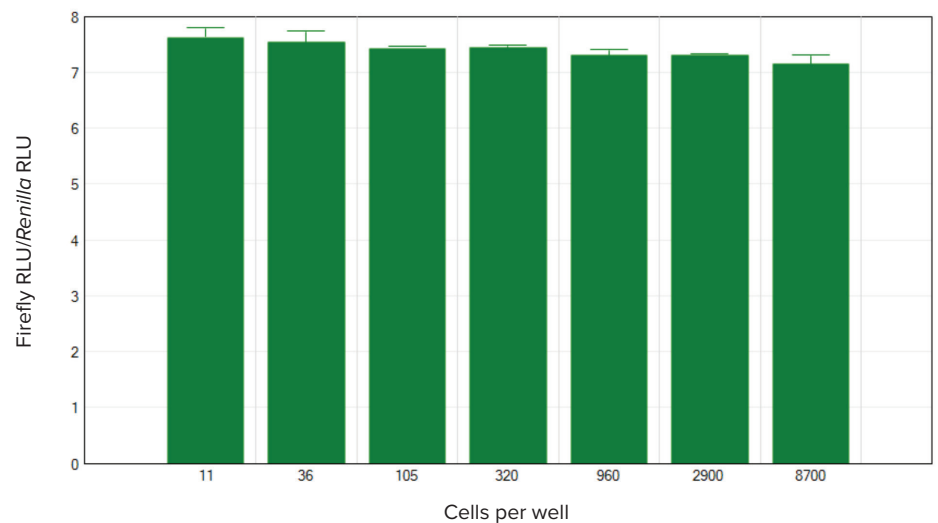


Figure 4. Normalized firefly luminescence units vs. cell concentration. SoftMax Pro Software automatically calculates and graphs luminescence values for firefly luciferase normalized to *Renilla* values. $N=3$ replicates at each cell number.

References

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