APPLICATION NOTE

Detect dual luciferase expression on the FlexStation 3 microplate reader

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Introduction

Reporter gene assays are important tools for studying gene expression associated with the activation of cellular pathways. Cells are transfected with a plasmid containing the reporter gene and a sequence of interest, typically a promoter or other transcriptional control element. When the promoter is activated, the reporter gene is expressed and its levels can be measured.

Firefly luciferase is a widely-used reporter gene. Its luminescent signal offers exquisite sensitivity compared to fluorescence or other methods. A second luminescent reporter, *Renilla* luciferase, is often used under the control of a constitutive promoter to normalize for sources of variability such as transfection efficiency and cell number. Since firefly and *Renilla* luciferases use different substrates, both can be measured in the same well using a dual luciferase detection assay.

The SpectraMax® DuoLuc Reporter Assay Kit enables highly sensitive quantitation of both firefly and *Renilla* luciferases in a microplate format. Addition of firefly working solution to a sample well initiates the firefly luminescence reaction, and subsequent addition of *Renilla* working solution simultaneously quenches the firefly and initiates the *Renilla* reaction.

The FlexStation® 3 Multi-Mode Microplate Reader can be used to run the SpectraMax DuoLuc assay in 96- and 384-well formats with high sensitivity and throughput. On the FlexStation 3 reader, the entire series of reactions is monitored in real time with simultaneous on-board reagent pipetting and luminescence detection (Figure 1).

Materials

- FlexStation 3 Multi-Mode Microplate Reader (Molecular Devices cat. #Flex3)
  - FlexStation 3 8-channel Pipettor Head (Molecular Devices cat. #0200-6182)
  - FlexStation 3 16-channel Pipettor Head (Molecular Devices cat. #0200-6183)
  - 96-well Black FlexStation Pipet Tips (Molecular Devices cat. #0900-0911)
  - 384-well Black FlexStation Pipet Tips (Molecular Devices cat. #9000-0764)
- 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)

Benefits

- **Higher assay throughput with column dispensing and simultaneous detection**
- **Detection of both firefly and *Renilla* luciferase expressions in as few as ten cells per well**
- **Preconfigured protocols for streamlined assay setup and fast results**

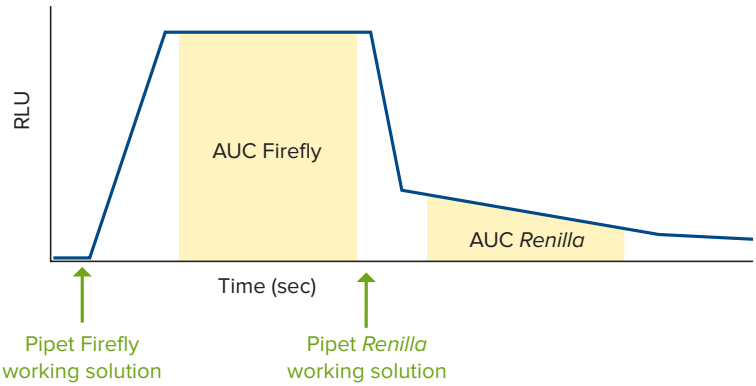

Figure 1. The reaction is monitored on the FlexStation 3 reader in real time as reagent additions occur. Area under the curve (AUC) for a specified portion of each phase of the reaction (firefly and *Renilla*) is calculated by the software.

Methods

Cell Transfection

HeLa cells were seeded at 2x10^5 cells per well in 6-well tissue-culture treated plates and incubated at 37°C/5% 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 µg/µL, and the pGL4.75[*hRluc*/CMV] *Renilla* luciferase expression vector was diluted to 100 ng/µL. Three tubes were set up as follows and mixed gently: 400 µL of Opti-MEM medium + 2 µL (2 µg) of pGL4.13[*luc2*/SV40] firefly luciferase expression vector + 2 µL (2 ng) of pGL4.75[*hRluc*/CMV] *Renilla* luciferase expression vector. To each tube, 6 µL of ViaFect reagent was added and the contents were gently mixed. Tubes were incubated for 10 minutes at room temperature to allow transfection complexes to develop. 200 µL of complex was added dropwise to each well of the 6-well plate, with gentle swirling to mix. Cells were returned to the incubator for 48 hours prior to processing and assay.

Preparation of Cell Lysates

Transfected cells in the 6-well plate were trypsinized, divided into ten aliquots per well, 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 µL 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 4 to 8700 cells per well. 20 µL of each concentration of cell lysate was pipetted into triplicate wells of a 96-well plate, and 10 µL of each concentration of cell lysate was pipetted into quadruplicate wells of a 384-well plate.

Luciferase assay setup

All kit components were thawed to room temperature. Firefly Substrate was reconstituted by adding 220 µL of water to one vial containing 2.2 mg of lyophilized substrate. Aquaphile™ Coelenterazine was reconstituted by adding 220 µL of water to one vial containing 440 µg of lyophilized substrate.

Parameter	96-well	384-well
Read mode	Luminescence	
Read type	Flex	
Wavelengths	All	
Integration time	200 ms	
Timing	2 minutes	3 minutes 40 seconds
Interval time	3.4 seconds	5.2 seconds
1st compound transfer	Add 100 µL of firefly solution Height = 50 µL Addition rate = 4 Addition time = 20 seconds	Add 25 µL of firefly solution Height = 25 µL Addition rate = 4 Addition time = 20 seconds
2nd compound transfer	Add 100 µL of *Renilla* solution Height = 150 µL Addition rate = 4 Addition time = 60 seconds	Add 100 µL of *Renilla* solution Height = 75 µL Addition rate = 4 Addition time = 70 seconds
Trituration	After 1st compound transfer: Height = 50 µL Cycle = 2 Volume = 50 µL After 2nd compound transfer Height = 100 µL	After 1st compound transfer: Height = 25 µL Cycle = 2 Volume = 25 µL After 2nd compound transfer Height = 25 µL
Data reduction	Firefly: 20 – 67 seconds *Renilla*: 68 – 120 seconds	Firefly: 20 – 80 seconds *Renilla*: 80 – 220 seconds

Table 1. FlexStation 3 microplate reader compound addition and assay parameters.

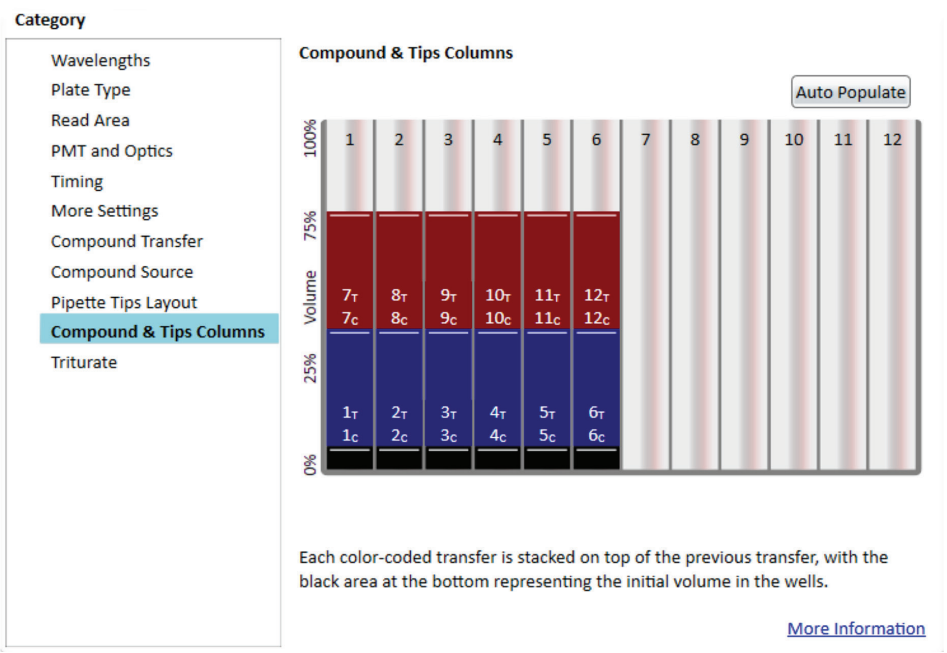

Figure 2. SoftMax Pro 7 Software user interface for FlexStation 3 microplate reader. The software's straightforward interface simplifies tip and compound column assignment.

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 µL of its respective substrate.

A preconfigured protocol in SoftMax® Pro Software was used with the parameters shown in Table 1. The software's graphical interface facilitated the setup of compound addition (Figure 2). The FlexStation 3 microplate reader is able to pipet reagent to an entire column of the plate and read these wells repeatedly for a defined amount of time using the Flex read type. A real-time kinetic trace is generated for each sample. Within each kinetic trace, the firefly and *Renilla* signals could be distinguished, and the area under the curve calculated for each (Figure 3).

Results

Firefly and *Renilla* luciferases were measured in transfected HeLa cells using the SpectraMax DuoLuc Reporter assay and FlexStation 3 microplate reader. The assay run in the 96-well format demonstrated great linearity and sensitivity. We were able to detect both firefly and *Renilla* luminescence from 8700 cells per well down to approximately 4 cells per well (Figure 4).

Assay performance was comparable for the 384-well format, with the same degree of linearity and detection down to approximately 8 cells per well (Figure 5).

Conclusion

The SpectraMax DuoLuc reporter assay, when combined with the FlexStation 3 microplate reader's built-in fluidics and luminescence detection, is a sensitive flash-type luminescent assay for accurately measuring gene expression in mammalian cells. The reader also provides higher assay throughput than dual injector-based systems due to its ability to dispense reagent to an entire column of the plate and read the wells of the column repeatedly for a selected total experimental time. SoftMax Pro Software's intuitive interface and preconfigured protocol allows users to quickly set up, measure, and analyze dual luciferase results.

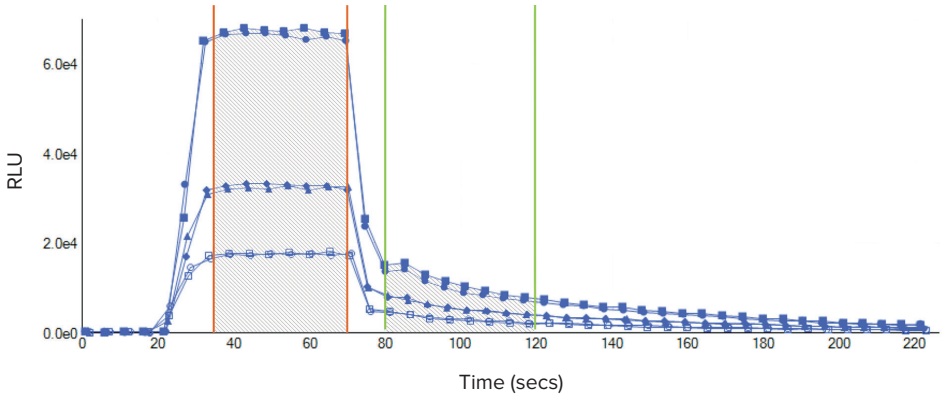

Figure 3. DuoLuc Kinetic Trace. The luminescent signal from the DuoLuc reporter assay was recorded over time to create a kinetic trace. Shown above are three different concentrations of cells assayed in a 384-well microplate format. Area under the curve for the firefly (35-70 seconds) and *Renilla* (80-120 seconds) regions of the kinetic trace are shown above. Reagent transfers occurred at 20 seconds and 70 seconds.

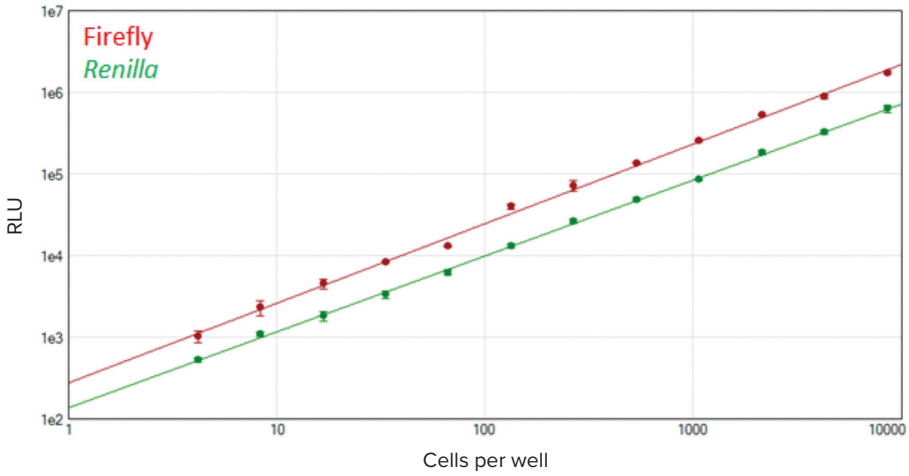

Figure 4. DuoLuc reporter assay in a 96-well format. The DuoLuc reporter assay was run in a 96-well plate using the FlexStation 3 microplate reader. Firefly (red) and *Renilla* (green) standard curves were plotted using a log-log curve fit in SoftMax Pro Software ($r^2 > 0.998$ for each). Three replicates were run for each dilution.

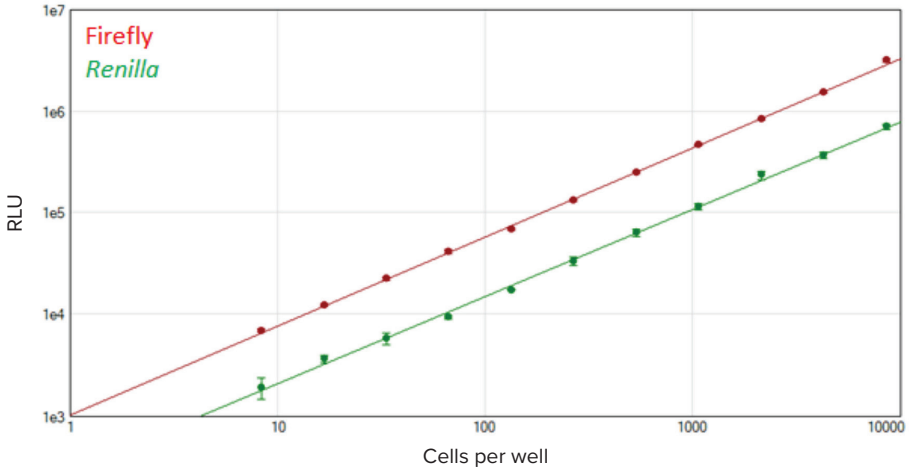

Figure 5. DuoLuc reporter assay ran in a 384-well format. The SpectraMax DuoLuc reporter assay was run in a 384-well plate. Firefly (red) and *Renilla* (green) standard curves were plotted using a log-log curve fit in SoftMax Pro Software ($r^2 > 0.998$ for each). Four replicates were run for each dilution.

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8/17 2124A
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