

Validation and Comparison of Single-Step Flash and Dual-Spectral Luciferase Reporter Gene Assays using the Synergy™ Line of Microplate Readers

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Introduction

Reporter assays have long been used to investigate regulatory mechanisms of gene expression in response to environmental stimuli in a variety of organisms. Reporter constructs rely on the insertion of a genetic regulatory element upstream of a reporter gene followed by insertion of the construct, generally a plasmid, into the target cell or organism (Figure 1).

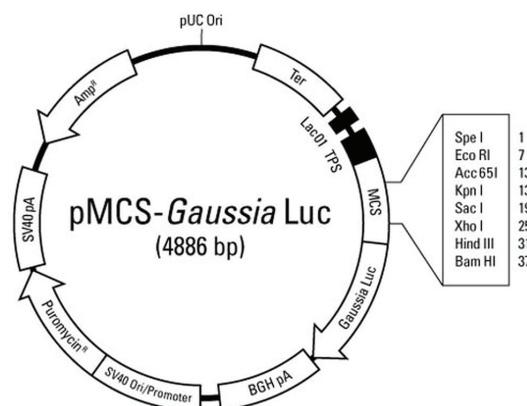


Figure 1. Thermo Scientific™ pMCS-Gaussia Luc reporter construct. The regulatory element of interest can be inserted into the multiple cloning site (MCS) upstream of the Luc reporter gene. Selective pressure can be applied by the use of Puromycin or Ampicillin in culture.

The introduction of luciferase-based reporter constructs provided a novel method of signal detection, the enzymatic production of light (luminescence), resulting in little background interference under a wide range of experimental conditions¹. In a luciferase reporter assay, the expression of luciferase is driven by the regulatory element and measurement of the luminescent signal allows quantification of the activity of factors affecting the signaling pathway under investigation (Figure 2)².

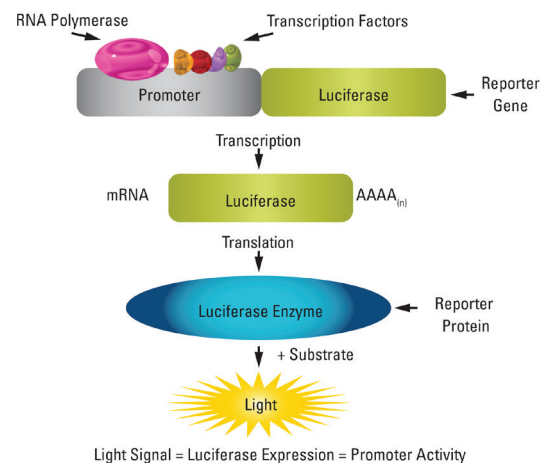


Figure 2. Schematic representation of a luciferase reporter assay.

Over the past several decades a diverse range of luciferase variants were discovered; many variants require a unique set of substrates and cofactors, while others require none, for light production. More importantly, the light generated can vary in regard to stability and brightness as well as the emission spectrum². The advent of multiple forms of luciferase has naturally lent itself to the development of assays utilizing multiple reporter constructs in a multiplexed fashion. Often dual-reporter assays combine the use of two different luciferases in a single organism or cells; one of which is used to investigate the experimental parameter (experimental reporter) and a second is used as an internal control to measure transfection efficiency, cell viability and cell plating precision for experimental normalization (normalization reporter)².

Here we investigate the performance of a new type of dual luciferase assay using two luciferases with spectrally resolvable light emissions in either 96- or 384-well density microplates using the Thermo Scientific Gaussia-Firefly Luciferase Dual Assay or Gaussia and Renilla Luciferase Flash Assay Kits, respectively. Assay optimization was performed and assay performance was compared across several BioTek Synergy™ Multi-Mode Microplate Readers configured with injectors using lysate containing expressed luciferase enzymes.

Materials and Methods

Luciferase Flash Assays

Renilla and Gaussia Luciferase Flash Assay Kits (Cat. # 16165 and 16159, respectively) and cell lysates from cells expressing Renilla or Gaussia luciferase and control cell lysate devoid of luciferase enzyme were a gift from the Protein Biology unit of Thermo Fisher Scientific (Rockford, IL). Assays were performed in solid, white 384-well microplates (Cat. #3705, Corning Inc. Life Sciences, Tewksbury, MA). The assay was performed as per the manufacturers' recommendations with the following modification for use in a 384-well microplate format. Briefly, the 100X Coelenterazine stock was aliquoted and stored at -80 °C, protected from light. A 1x Coelenterazine working solution was prepared from the stock solution on the day of use by diluting 50 µL into 5 mL of the appropriate Flash Assay Buffer. For optimization of reader setting, the cell lysates were diluted 1x, 1,000x (E-3x), or 10,000x (E-5x) in cell lysis buffer. Cell lysates were also subjected to an 11-point half-log dilution series into lysis buffer including a control consisting of lysis buffer alone. For all assays, the microplate reader injectors were primed with 1.2 mL of working solution just prior to performing the assay. Each assay well containing 5 µL of cell lysate or lysis buffer alone received 25 µL of working solution using the microplate reader injectors immediately before detection of light output. The assay protocol was run in well-mode from the Gen5™ Data Analysis Software (BioTek Instruments, Inc., Winooski, VT) with the parameters outlined in Table 1 and data exported to either Microsoft Excel (Redmond, WA) or GraphPad Prism (La Jolla, CA) for analysis.

Gaussia-Firefly Luciferase Dual Assay

Gaussia-Firefly Luciferase Dual Assay Kit (Cat. #16182) and cell lysates from cells expressing Gaussia or Firefly luciferase or control cell lysate devoid of luciferase enzyme were a gift from Thermo Fisher Scientific (Rockford, IL). Assays were performed in solid, white 96-well microplates (Cat. #3912, Corning Inc. Life Sciences, Tewksbury, MA). The assay was performed as per the manufacturers' recommendations with the following modification for use in a 96-well microplate format. Briefly, the lyophilized D-luciferin pellet was resuspended in the appropriate volume of Gaussia-Firefly Dual Assay Buffer (0.5 mL) resulting in a 100x stock.

The 100x stock was aliquoted and stored at -20 °C protected from light. Coelenterazine stock solution was prepared as described above. A working solution was prepared from the stock solutions on the day of use by diluting 50 µL of reconstituted D-Luciferin and 50 µL of Coelenterazine stock solution into 5 mL of Gaussia-Firefly Dual Assay Buffer. The Gaussia luciferase cell lysate was subjected to a half-log dilution series in lysis buffer including a control consisting of lysis buffer alone. The Firefly luciferase cell lysate was diluted 2-fold for use as an internal control. Gaussia lysate or lysis buffer was mixed at a 1:1 (v/v) ratio with the firefly luciferase lysate in a total volume of 20 µL. The microplate reader injectors were primed with 1.2 mL of working solution just prior to performing the assay. Each assay well containing 20 µL of cell lysate or lysis buffer received 50 µL of working solution using the microplate reader injectors immediately before detection of light output. The assay protocol was run and analyzed as described above.

A.

Flash Assay Type	Renilla	Gaussia
Mode	Lum	Lum
Excitation	Plug	Plug
Emission	530/25	485/40
Gain	100	100
Integration time	1 sec	1 sec
Delay	0 msec	0 msec

B.

	H1	Neo	H4
96-well microplate	7	6	1
384-well microplate	9.25	8.25	1

C.

Synergy H1		
Mode	Lum	Lum
Excitation	Plug	Plug
Emission	485/20	640LP
Gain	225	225
Integration time	1 sec	1 sec
Delay	0 msec	0 msec
Synergy H4		
Mode	Lum	Lum
Excitation	Plug	Plug
Emission	485/20	640LP
Gain	225	225
Integration time	1 sec	1 sec
Delay	0 msec	0 msec
Synergy Neo		
Mode	Lum	Lum
Excitation	Plug	Plug
Emission	485/20	640LP
Gain	225	225
Integration time	0.2 sec	
Delay	0 msec	0 msec

Table 1. Readers parameters. A) Reader parameters used for performing the luciferase flash assays in 96-well format; B) optimized read heights used for each assay format; and C) reader parameters used for performing the dual luciferase assay in 384-well format.

Results and Discussion

Optical probe height (distance from the microplate to the instrument optics) can have a considerable effect on the signal to background ratio which becomes increasingly important as assay volumes are miniaturized in higher density microplate formats. The signal generated from the flash assay using highly expressed luciferases in cell lysate was stable for an adequate length of time to determine the optimal probe height for each reader. Optimization of the probe Z-height was performed using the read height auto-adjust function in the Gen5 software on the Synergy H1 and Synergy Neo (Figure 3) resulting in optimal signal sensitivity at heights of 9.25 and 8.25 mm, respectively. The Synergy H4 Z-height was optimized by manual methods (data not shown) by incrementing the read height in successive read steps. It was shown that the minimal probe height of 1 mm was the most sensitive.

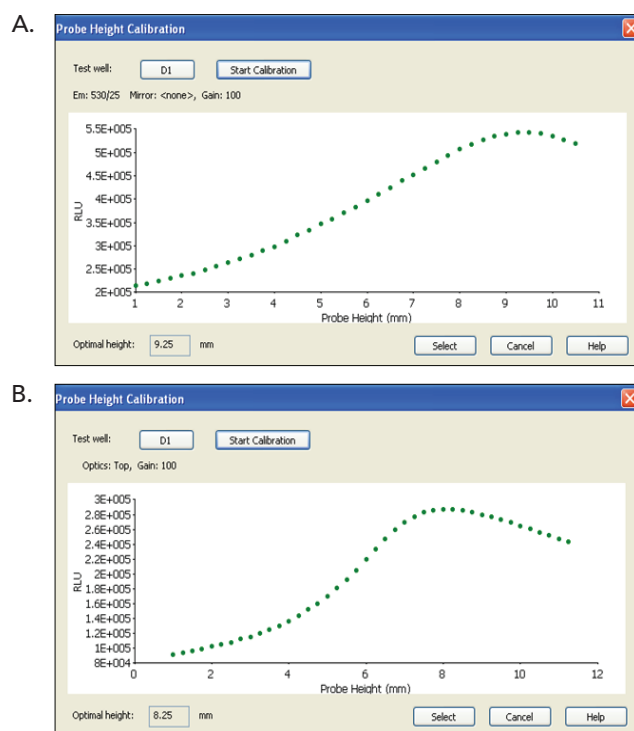


Figure 3. Automated Z-height optimization. Representative data from the Thermo Scientific Gaussia Luciferase Flash Assay showing signal intensity curves as a function of probe Z-height for assays performed in a 384-well density microplate read on **A)** Synergy H1 and **B)** Synergy Neo.

Once determined, the optimal probe Z-height was used for determination of an appropriate gain setting for optimal signal detection and to account for variability in expression levels and signal intensity seen between the various luciferase variants. The optimal gain setting was determined as that which resulted in an optimal S/B ratio while insuring signal intensity remained below the photomultiplier tube (PMT) saturation threshold (overflow) for undiluted cell lysate (1x). This process was repeated for both 96-well and 384-well microplate densities.

Luciferase Flash Assays

Renilla and Gaussia luciferase lysates were subjected to a half-log dilution scheme to investigate reader performance using the optimized reader parameters shown in Table 1. Nearly identical read parameters were used for all three instruments tested with the exception of the probe height and a much shorter integration time of 0.2 seconds used for the HTS instrument, Synergy Neo. Data was plotted as log-log with relative luminescence units plotted on the y-axis and fold-dilution plotted on the x-axis. The data was analyzed using log-log non-linear regression analysis in GraphPad Prism software to investigate relative linearity (Figure 4). It is obvious that lysate containing expressed Renilla Luciferase (Figure 4A) shows excellent linearity across all readers tested with a dynamic range spanning five decades. Gaussia Luciferase demonstrates satisfactory linearity, although there is some additional signal variability with Synergy H1 in the final three decades of the dilution series.

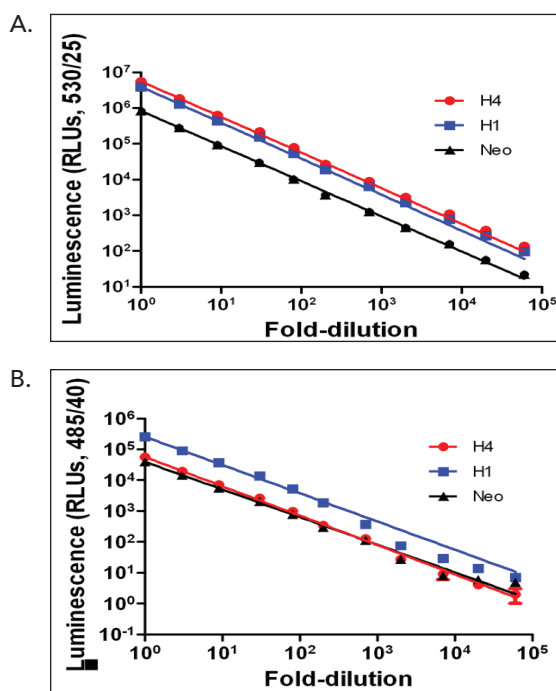


Figure 4. Renilla and Gaussia Luciferase Flash Assays. **A)** Renilla and **B)** Gaussia Luciferase containing lysates were subjected to a half-log titration in lysis buffer. Data shown as luminescent signal (RLUs) versus fold-dilution across a dynamic range spanning five orders of magnitude.

Gaussia-Firefly Luciferase Dual Assay

The luciferase dual assays rely on the ability to spectrally resolve a pair of distinct luciferase enzymes producing light at different wavelengths. A typical application of a dual spectral luciferase reporter gene assay allows simultaneous monitoring of luciferase activity from a reporter plasmid as well as from a second luciferase control plasmid providing a means to normalize well-to-well variability. While any number of luciferases can be spectrally resolved, Gaussia and red firefly luciferase have very little spectral overlap (Figure 5). By careful selection of the appropriate filters, the signal from each variant can be read either sequentially or simultaneously, depending on reader capabilities, after addition of the one-step detection reagent containing the appropriate cofactors and substrates. As shown in figure 6, the recommended filter ranges are a 485 ± 20 nm band-pass (BP) filter for Gaussia light output and a 640 nm long-pass (LP) filter for red firefly output ensuring minimal light crossover.

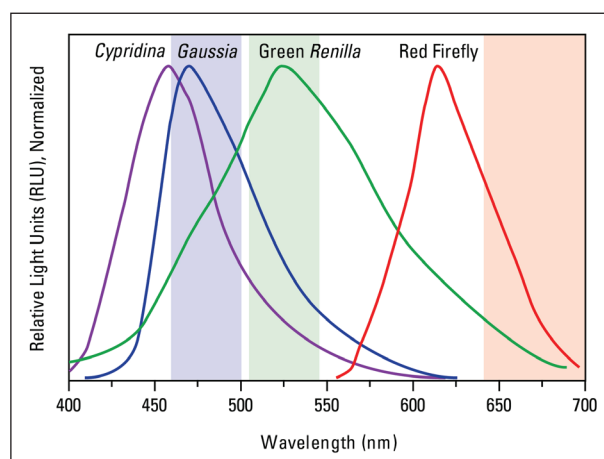


Figure 5. Gaussia-Firefly Dual Luciferase Assay emission spectra profile. Shaded areas represent the recommended filter ranges with $\lambda_{\text{max}} = 485$ nm and 613 nm for Gaussia and red firefly luciferases, respectively.

A typical dual spectral luciferase reporter assay protocol is summarized below (Figure 6). To mimic assay conditions, such as an inhibitory dose response of a compound, Gaussia lysate was diluted (experimental reporter) and added 1:1 (v/v) to red firefly lysate (normalization control) held at a constant concentration as described above. The assays were initiated by a single reagent injection and the dual luminescent signals were read either sequentially, on Synergy H1 and H4, or simultaneously using the dual-emission detection capabilities of the Synergy Neo.

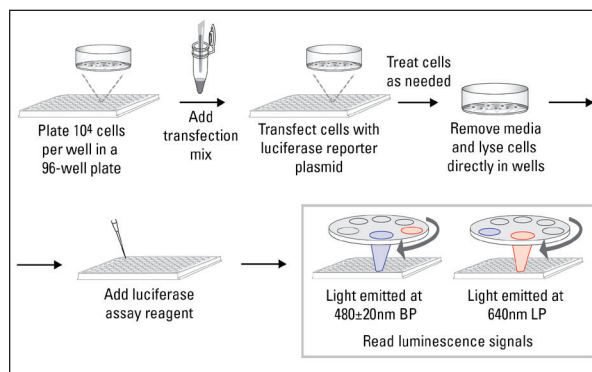


Figure 6. Protocol summary for the Thermo Scientific Gaussia-Firefly Luciferase Dual Assay Kit. This is a one-step procedure for spectrally resolving two luciferases. By including both substrates in a single assay reagent, two luciferase activities can be measured simultaneously in a sample by filter-based detection.

It is evident that the red firefly luciferase signal shows good linearity regardless of the Gaussia luciferase concentration across the range of concentrations tested on all readers (Figure 7). The Gaussia luciferase trends as discussed above were again evident.

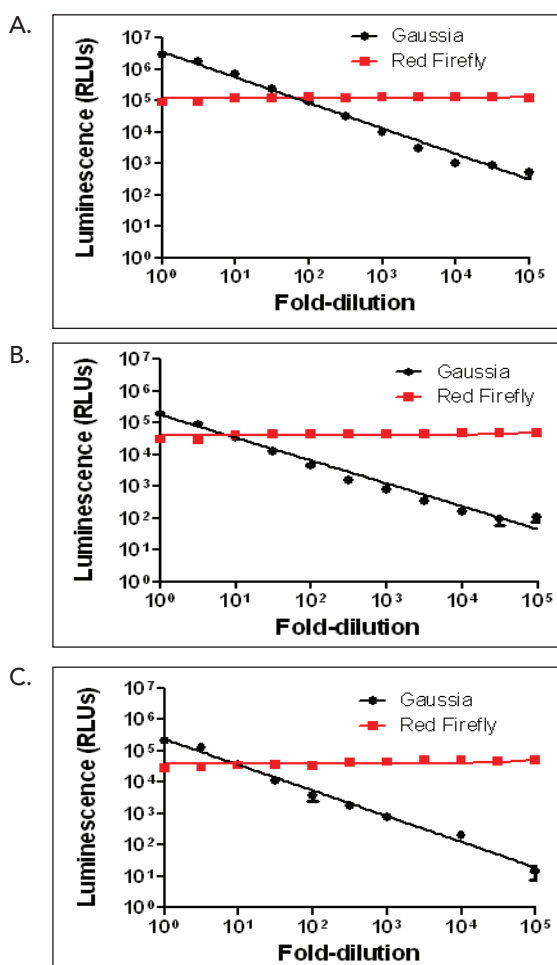


Figure 7. Gaussia-Firefly Luciferase Dual assay. The Gaussia-Firefly Luciferase Dual Assay Kit was used to determine the activity from each reporter on a variety of Synergy readers. The assay was performed as a single step assay mimicking a typical dual reporter assay. A) Synergy H4 B) Synergy H1 and C) Synergy Neo.

Conclusion

Luciferase-based reporter assays continue to play an important role in cell-based assays by providing a highly sensitive method to investigate complex signaling events. The ability to multiplex luciferase variants allows normalization of data by monitoring cell viability, transfection efficiencies and plating variability. Here we have shown the ability to successfully perform Pierce Luciferase based assays across a range of Synergy microplate readers with comparable results in several microplate formats. In addition, we have shown the ability to perform a luciferase dual assay by either performing sequential reads of spectrally resolvable luciferases or simultaneous reads by taking advantage of the Synergy Neo with its multiple parallel detectors. The optimal reader settings and filter sets have also been described resulting in the ability to detect luciferase concentrations ranging over 5 orders of magnitude.

References

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