Application Note:
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Validated Luminometric DLR™ Reporter Gene Assay with Thermo Scientific Varioskan® Flash

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One of the most common luminometric assays is Dual Luciferase® Reporter Assay from Promega corp. This application note shows how this assay is performed with Varioskan Flash multitechnology microplate reader. The note includes the performance of the normal DLR assay and results from some tests showing important aspects of the basic behaviour and performance of the DLR™ chemistry. This paper describes the overall efficiency of the Varioskan Flash in the measurement of DLR assay.

Introduction

Luminescent reporter gene systems are very commonly used to study gene expression and regulation. One can use reporter genes to study receptor activity, transcription factors, intracellular signalling, mRNA processing or protein folding. In dual reporter gene assays one simultaneously expresses and measures two different reporter enzymes. One reporter enzyme is referred as “experimental” reporter and it responds to the effects of the experimental conditions. Another reporter enzyme is normally referred as “control” and it works as an internal control that is used to eliminate experimental variability for example in cell viability, transfection or cell lysis efficiency or even in pipetting volumes.

The Dual-Luciferase Reporter (DLR) Assay System from Promega corp. is probably the most common dual reporter gene system. This system uses two different luciferase genes from distinct origin: one from North American firefly (Photinus pyralis) and one from Renilla sea pansy (Renilla reniformis) (1-3). This dual assay includes sequential measurement of these two luciferases. The firefly luciferase activity is measured first by adding Luciferase Assay Reagent II (LAR II). After the signal has been quantified, firefly reaction is quenched and the Renilla luciferase reaction is simultaneously initiated by adding Stop&Glo® Reagent (4). Both luminescence signals have stable light output making the assay easy to perform. Assay sensitivities with both luciferases are in attomole levels and they both give linear response over a range of more than six orders of magnitude, making it possible to measure extremely low gene expression levels from cell lysates.

Materials required

- Varioskan Flash microplate reader with Varioskan LumiSens luminometric module, two automatic dispensers and SkanIt software, Thermo Scientific (#5250030 or #5250040 equipped with #5250500 and 2x #5250510)
- Dual-Luciferase Reporter Assay System, Promega corp.(#E1980, #E1910 or #E1960)
- Quantilum recombinant firefly luciferase, Promega corp. (#E1701)
- Renilla reniformis luciferase, LUX Biotechnology Inc. (#NL-RRLUC100)
- Microlite 1+, 96 well white microtiter plate, Thermo Scientific (#7571)
- Pipettes and tips with volumes 10 – 5000 ul

Experimental protocols

Reagent preparation

Luciferase assay reagent (LAR II), Stop&Glo reagent and 1X Passive Lysis Buffer (PLB) were prepared according to the instructions in the kit. Quantilum recombinant firefly luciferase (1 mg) was dissolved in 3.28 ml of 1X PLB buffer with 0.1% Gelatine to get $5X10^{-9}$ mol/ml stock solution. Renilla reniformis luciferase, (0.1 mg) was dissolved in 5.56 ml of 1X PLB buffer with 0.1% Gelatine to get $5X10^{-10}$ mol/ml stock solution. Both stock solutions were divided into small aliquots and stored in -70 C freezer. All required luciferase samples with final concentrations between 10^{-20} – 10^{-13} mol/well (0.01 - 100 000 amol/well) were prepared from the stock solutions by diluting the stock solutions with 1X PLB.

Instrument preparation

Varioskan Flash instrument used here has two automatic dispensers. Dispenser 1 was primed with LAR II Reagent and was set to dispense 100 ul with “High” dispensing speed. Dispenser 2 was primed

with Stop&Glo Reagent and also set to dispense 100 ul with "High" speed. Instrument's temperature setting was kept in "Ambient" to keep the unit in ambient temperature of 22 C.

Preliminary tests

Luminometric spectra

Luminometric spectra of both firefly and Renilla luciferase reactions were measured with spectral scanning luminescence option using 10 000 amol/well as final luciferase concentration for both firefly and Renilla luciferase. Samples of both luciferases were added as 20 ul aliquots with replicates into the same wells of 96 well white Thermo Microlite 1+ plate. Varioskan Flash was then programmed to perform following measurement:

- STEP 1: Well loop step, Execution type "by well", Well count 1.
Sets the instrument to perform the following actions well by well, instrument executes all actions to one well before proceeding to the next well.
- STEP 2 (inside well loop): Dispensing step, Dispenser 1, Dispensing volume 100 ul, Dispensing speed "High", Dispensing position "Luminometric 1", Tip priming "No".
Dispenses 100 ul of LAR II Reagent into the well with high speed (predefined "High speed" option with Varioskan uses the speed of 40% from the maximum possible dispensing speed (correlates to about 400 ul/second).
- STEP 3 (inside well loop): Measurement step, Luminometric spectral scanning, wavelength area 450 – 700 nm, Wavelength step 1 nm, Dynamic range "AutoRange", Measurement time 500 ms, Lag time 2 s.
Measures firefly luciferase spectra from the selected wavelength area with one nanometer steps. Before beginning the measurement, a two second lag time period is waited to let the firefly signal to be generated.
- STEP 4 (inside well loop): Dispensing step, Dispenser 2, Dispensing volume 100 ul, Dispensing speed "High", Dispensing position "Luminometric 2", Tip priming "No".
Dispenses 100 ul of Stop&Glo Reagent into the well with high speed.
- STEP 5 (inside well loop): Measurement step, Luminometric spectral scanning, wavelength area 400 – 600 nm, Wavelength step 1 nm, Dynamic range "AutoRange", Measurement time 500 ms, Lag time 2 s.
Measures Renilla luciferase spectra from the selected wavelength area with one nanometer steps. Before beginning the measurement, a two second lag time period is waited to let the Renilla signal to be generated.
Schematic structure of the program is shown in Figure 1.
The assay plate was placed into the instrument's plate carrier and full program was executed. After the measurement, all data was exported from the SkanIt software as an Excel file using SkanIt's "Organized export" function and data analysis was performed with Excel.

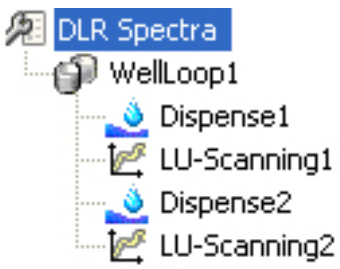

Figure 1. Structure of the measurement protocol used to measure luminescence spectra.

Quenching efficiency of the firefly luciferase

One very important step in the DLR assay is that firefly luciferase signal must be efficiently quenched when Renilla luciferase signal is initiated with Stop&Glo Reagent. This quenching efficiency was tested with firefly luciferase samples without Renilla luciferase. Very high firefly luciferase concentration of 100 000 amol/well was used in this quenching efficiency test to ensure sufficient quenching efficiency over whole luciferase concentration range. Quenching efficiency was tested using kinetic measurement mode of the instrument so that development of the firefly luciferase signal could be revealed in very detail. Samples of firefly luciferase were added as 20 ul aliquots with 16 replicates into the wells of 96 well white Thermo Microlite 1+ plate. Varioskan Flash was programmed as follows:

- STEP 1: Well loop step, Execution type "by well", Well count 1.
Sets the instrument to perform the following actions well by well.
- STEP 2 (inside well loop): Kinetic loop step "KineticLoop1", Readings 60, Interval 0 s.
This sets the instrument to perform kinetic assay with 60 kinetic points. Kinetic reading is performed as fast as possible without any interval time between the readings.
- STEP 3 (inside kinetic loop 1): Dispensing step, Dispenser 1, Dispensing volume 100 ul, Dispensing speed "High", Dispensing position "Luminometric 1", Tip priming "No", Dispense at reading 1.
Dispenses 100 ul of LAR II Reagent into the well with high speed. Dispensing is performed

simultaneously with the first kinetic reading point.

• STEP 4 (inside kinetic loop 1): Measurement step, Luminometric measurement, Luminometric optics “Normal”, Dynamic range “AutoRange”, Measurement time 200 ms.
Performs kinetic measurement with the sampling speed of 5 points/second (200 ms measurement time), Total kinetic measurement time is 12 seconds (5 points/second and totally 60 points). This steps measures firefly luciferase signal without quenching.

• STEP 5 (inside well loop): Kinetic loop step “KineticLoop2”, Readings 60, Interval 0 s.
Again, this sets the instrument to perform kinetic assay with 60 kinetic points. Kinetic reading is performed as fast as possible without any interval time between the readings.

• STEP 6 (inside kinetic loop 2): Dispensing step, Dispenser 2, Dispensing volume 100 ul, Dispensing speed “High”, Dispensing position “Luminometric 2”, Tip priming “No”, Dispense at reading 1.
Dispenses 100 ul of Stop&Glo Reagent into the well with high speed simultaneously with the first kinetic reading of the kinetic loop 2.

• STEP 7 (inside kinetic loop 2): Measurement step, Luminometric measurement, Luminometric optics “Normal”, Dynamic range “AutoRange”, Measurement time 200 ms.
Performs kinetic measurement with the sampling speed of 5 points/second (200 ms measurement time), Total kinetic measurement time is 12 seconds (5 points/second and totally 60 points). This step measures development of the quenching after Stop&Glo Reagent has been added.
Step structure of the protocol is shown in Figure 2.

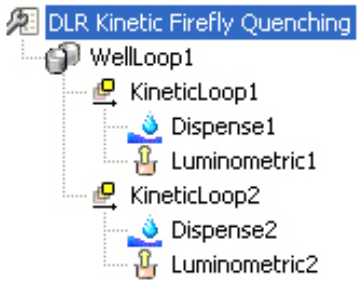

Figure 2. Protocol step tree for the kinetic measurement of firefly luciferase quenching.

When measurements were done all data was transferred to Excel same way as previously. Average kinetic curve was drawn with the standard deviation of the replicates and quenching efficiency for each sample was calculated. For the calculations, luciferase signal before quenching was integrated from 2 s to 12 s and signal after quenching from 14 s to 24 s from the kinetic curves. Quenching efficiency was calculated as a ratio between these before and after signals. In addition, Z’-prime value (5) between the signals before and after the quenching was calculated from the data.

Consistency of the measurements
Measurement consistency and signal ratio linearity are important factors in DLR assay. This was tested by making two mixtures of firefly and Renilla luciferases. One mixture contained 1000 amol/20 ul firefly and 10 amol/20 ul Renilla luciferase (High firefly and low Renilla conc.), and another was the opposite, 10 amol/20 ul firefly and 1 000 amol/20 ul Renilla luciferase. (Low firefly and high Renilla conc.). A 20 ul aliquot of both mixtures was added into the plate with 16 replicates. Following assay protocol was created for Varioskan Flash:

• STEP 1: Well loop step, Execution type “by well”, Well count 1.
Sets the instrument to perform the following actions well by well.

• STEP 2 (inside well loop): Dispensing step, Dispenser 1, Dispensing volume 100 ul, Dispensing speed “High”, Dispensing position “Luminometric 1”, Tip priming “No”.
Dispenses 100 ul of LAR II Reagent into the well with high speed.

• STEP 3 (inside well loop): Measurement step, Luminometric measurement, Luminometric optics “Normal”, Dynamic range “AutoRange”, Measurement time 10 000 ms, Lag time 2 s.
Measures the signal with 10 s integration time after 2 s waiting timer before the measurement starts.

• STEP 4 (inside well loop): Dispensing step, Dispenser 2, Dispensing volume 100 ul, Dispensing speed “High”, Dispensing position “Luminometric 2”, Tip priming “No”.
Dispenses 100 ul of Stop&Glo Reagent into the well with high speed.

• STEP 5 (inside well loop): Measurement step, Luminometric measurement, Luminometric optics “Normal”, Dynamic range “AutoRange”, Measurement time 10 000 ms, Lag time 2 s.
Measures the signal with 10 s integration time after 2 s waiting timer before the measurement starts.
The protocol structure used in the test is shown in Figure 3.

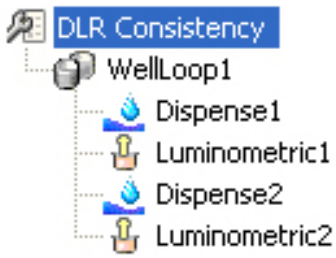

Figure 3. Protocol step structure for the consistency measurements.

After the measurement, data was exported to Excel and following basic statistical parameters were calculated: average, standard deviation (based on the sample) and CV%. In addition, high concentration to low concentration ratio was calculated.

Sensitivity and dynamic range of the DLR assay

When all preliminary tests have been performed, sensitivity and dynamic range on the determination of both luciferases were measured. For that, a dilution series between 0.01 - 100 000 amol/10 ul was prepared from both luciferases and 10 ul aliquots were pipetted into white microplate so that same concentration of both luciferases was combined within the well. Each concentration was measured with 8 replicates and 16 replicates of assay blanks (1 x PLB) were used. Resulting plate layout is shown in the Table I. DLR sensitivity measurements were performed using automatic reagent dispensing with the same assay protocol that was used in consistency test. After measurement were finished all data was again exported to Excel. Calibration curves were drawn with linear regression analysis and sensitivity limits were calculated using IUPAC 3*SD method (10). Dynamic range was calculated from sensitivity value and maximum value in this assay, therefore dynamic range does not correlate to the dynamics of the instrument but only to this test. Robustness of the assay was estimated using Z'-prime values that were calculated from the calibration data.

Results and Discussion

Luminometric spectra

Luminometric spectra of both firefly and Renilla luciferase reaction was measured to ensure correct measurement conditions. Spectra are very sensitive to environmental changes; therefore changes in assay conditions could well be detected as a change in either shape or the peak values of the spectra. As seen from the Figure 4, resulting spectra show very typical form for these assays. In addition, both maximum emission peak wavelengths and widths of the peaks given in Table II are very representative for these luciferase reactions. Firefly luciferase light emission shows maximum at 562 nm and luminescence peak has about 80 nm half bandwidth. These values are identical to the previously reported data (6-7). Similarly, Renilla emission spectra is completely similar to earlier reported data with 486 nm emission maximum and same 80 nm half bandwidth (8-9).

Quenching efficiency of the firefly luciferase

Quenching efficiency of firefly reaction after Stop&Glo reagent addition was tested using kinetic follow up of the luminescence signal. Average kinetic curve of the quenching reaction is shown in Figure 5. and individual quenching efficiencies of replicate samples with their statistics is shown in Table III.

As seen in the Figure 5., firefly signal reaches its stable level in less than one second after firefly substrate is added. This requires very efficient high speed automatic dispensing that produces also efficient complete mixing of the sample and the reagent. Both even higher and clearly lower dispensing speeds were addition-

Table I. Plate layout used to determine DLR sensitivity and dynamic range.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank			0.01	0.1	1.0	10	100	1 000	10 000	100 000
B	Blank	Blank			0.01	0.1	1.0	10	100	1 000	10 000	100 000
C	Blank	Blank			0.01	0.1	1.0	10	100	1 000	10 000	100 000
D	Blank	Blank			0.01	0.1	1.0	10	100	1 000	10 000	100 000
E	Blank	Blank			0.01	0.1	1.0	10	100	1 000	10 000	100 000
F	Blank	Blank			0.01	0.1	1.0	10	100	1 000	10 000	100 000
G	Blank	Blank			0.01	0.1	1.0	10	100	1 000	10 000	100 000
H	Blank	Blank			0.01	0.1	1.0	10	100	1 000	10 000	100 000
					Concentration of both luciferases (amol/well)							

Table II. Spectral characteristics of luciferases.

Sample	Emission maximum (nm)	Half bandwidth (nm)
Firefly luciferase	562	77
Renilla luciferase	486	80

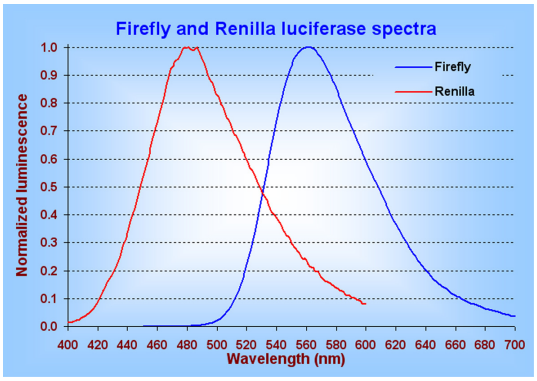

Figure 4. Spectra of firefly and Renilla luciferase luminescence reaction.

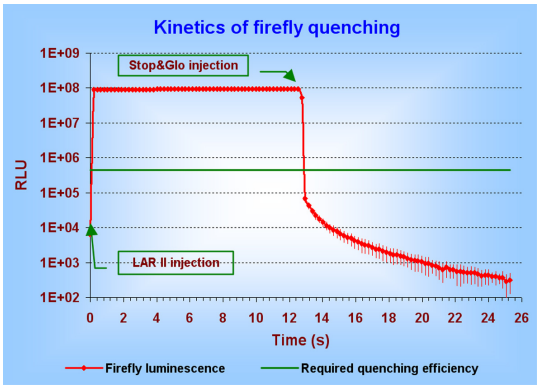

Figure 5. Kinetics of firefly luminescence quenching.

Table III. DLR quenching efficiency of firefly luminescence after Stop&Glo addition.

Sample	Firefly signal (RLU)	Quenched firefly signal (RLU)	Quenching efficiency
1	4 480 048 400	65 890	67 990
2	4 458 537 500	135 480	32 910
3	4 511 674 700	76 220	59 190
4	4 465 273 200	94 970	47 020
5	4 441 976 100	129 350	34 340
6	4 569 162 100	227 310	20 100
7	4 432 087 200	219 990	20 150
8	4 421 412 200	125 300	35 290
9	4 511 645 100	127 960	35 260
10	4 503 712 300	120 260	37 450
11	4 472 800 300	184 120	24 290
12	4 465 530 400	188 830	23 650
13	4 392 547 800	102 350	42 920
14	4 554 842 700	97 160	46 880
15	4 379 656 500	93 420	46 880
16	4 554 861 600	97 270	46 830
Average:	4 475 985 500	130 370	34 330
Z'-prime	0.96		

Table IV. Consistency of DLR assay.

	Average signal (RLU)	Standard deviation	CV%	High signal / low signal ratio
Firefly, 1 000 amol/well	1 133 825	25 949	2.3	102.5
Firefly, 10 amol/well	11 063	442	4.0	
Renilla, 1 000 amol/well	615 547	12 109	2.0	99.5
Renilla, 10 amol/well	6 189	217	3.5	

ally tested. Higher speed did not improve the signal generation but caused clear splashing problems and lower speed gave remarkable delay in signal generation (detailed data not shown). Therefore optimal dispensing speed with sufficient efficiency but without spilling or splashing is important requirement of the DLR assay. After the signal has reached the maximum level it is very stable over whole about 10 second follow up period. When Stop&Glo reagent with chemical quencher of the firefly reaction is added, the firefly signal needs to be decreased very rapidly. This is also achieved with high speed dispensing using Varioskan Flash automatic dispenser. The firefly signal is decayed after Stop&Glo addition in less than half of the second into the required level of 10 000 times decrease in the firefly luminescence. The signal was still decreasing during the whole measurement period of 12 seconds. Integrated signals from the time windows where DLR assay is normally measured (10 second integration time with two second delay after the dispensing) showed very efficient quenching with average quenching efficiency of over 34 000 times. Individual quenching efficiencies of the replicates showed little variations but efficiency was always clearly over the requirements. Calculated Z'-prime value for the quenching also shows very good separation of unquenched and quenched signal.

Consistency of the measurements

Assay consistency was tested with two luciferase mixtures, one with high firefly and low Renilla luciferase concentration and another with high Renilla and low firefly luciferase concentration, both with 100 times concentration difference between high and low. Such concentration levels were selected that they both are located in the middle part of the total concentration range of the assay. Collected results of these measurements are shown in Table IV. As seen, all four luciferase samples were measured with acceptable standard deviation and CV%. In addition, correct signal ratio of one hundred were achieved with both firefly and Renilla samples, proving that both luciferases were measured independently without interfering effect of another luciferase.

Sensitivity and dynamic range of the DLR assay

DLR assay sensitivity and dynamic range were determined with normal DLR assay protocol. Dilution series of both luciferases were mixed in the microplate and firefly and Renilla activities were measured according to standard protocol. Resulting standard curves are shown in Figure 6.

Both luciferase standard curves showed a very good linearity over the whole measurement range from 0.01 to 100 000 amol/well. Theoretical sensitivity of the assay for both luciferases was calculated using common IUPAC 3*SD method. In this method, a concentration point is calculated where the calibration curve crosses a line formed by the sum of assay blank plus three times

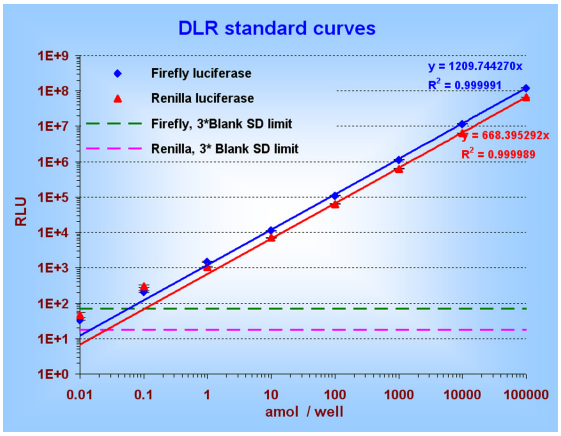

Figure 6. Firefly and Renilla luciferase standard curves from the DLR assay.

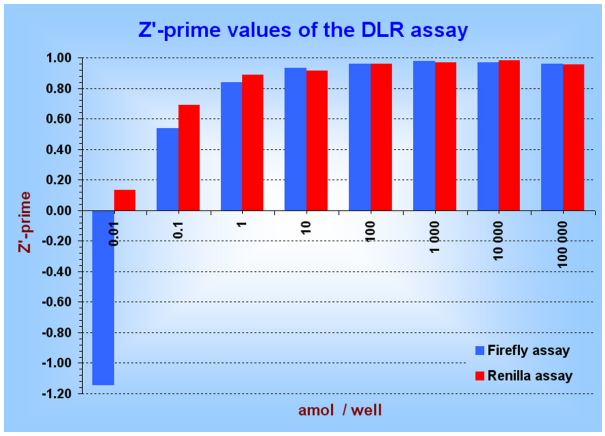

Figure 7. Z'-prime values of DLR assay with different luciferase concentrations.

Table V. DLR assay sensitivity and dynamic range.

Luciferase	Theoretical sensitivity (amol/well)	Dynamic range (orders of magnitude)
Firefly	0.06	6.2
Renilla	0.03	6.6

blank standard deviation. This concentration is considered to be the lowest that is possible to be statistically separated from the background. Dynamic measurement range for the DLR assay was calculated from the highest value of the calibration curve and sensitivity limit. Both sensitivity and dynamic range values are shown in Table V.

As seen from the table, DLR assay with Varioskan Flash can easily detect sub attomolar amounts of both firefly and Renilla luciferases and both have about the same sensitivity limit. Both assays also offer very wide over six orders of magnitude dynamic range. It is important to notice that standard curves are totally linear up to the highest standard used, without any notable plateau effect. Therefore, the real dynamic range could well be clearly larger because neither the instrument nor the assay chemistry has reached yet their upper linear detection limit in this test. Robustness of the DLR assay was estimated using Z'-prime analysis. Collected Z-prime values of all tested concentrations are shown in Figure 7. Z'-prime values confirm what was already seen from the theoretical sensitivities, the lowest calibrator of 0.01 amol luciferase clearly falls out of the measurement range and Z'-prime values of 0.1 amol standard are just in the area where assay can be considered reliable (for firefly and Renilla Z'-prime values are 0.54 and 0.69, respectively). With higher amounts of luciferase the assay gave very good Z'-prime values over 0.85, proving both reliability and robustness of the DLR assay.

Conclusions

Varioskan Flash gives in DLR assay very reliable, reproducible and robust results, and efficient high speed dispensing ensures fast quenching of the firefly signal. Additional features of Varioskan Flash, like luminometric spectral scanning or flexible kinetic follow up of the reaction, give unique possibilities to ensure optimal performance of the assay and useful possibilities for the quality control of the assays. Varioskan Flash with high sensitivity LumiSens luminometric module and with automatic dispensers is a perfect combination for performing DLR assays.

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