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Orion II Microplate Luminometer Simplicity 4

Dual Luciferase Reporter Assay (Promega)

Dual reporter systems are used to normalize reporter gene expression in transient transfection experiments. In this experimental setup the reporter enzyme quantifies the expression of the gene that is studied, whereas a control enzyme is used as an internal standard.
The ratio of the respective light units describes the reporter gene activity corrected with respect to transfection efficiency in mammalian cells. This method improves reliability and precision of transcriptional analysis.
In the Dual Luciferase Repoter™ Assay system both enzymes are luciferases, the reporter enzyme is the luciferase of the American firefly (*Photinus pyralis*), whereas the control enzyme is a *Renilla* luciferase (*Renilla reniformis*). The luminescence of both enzymes can be selectively detected and measured in a single sample, because they follow different biochemical mechanisms.
The reaction of the firefly luciferase is initiated by adding the Luciferase Assay Reagent II. In a second step quenching of the firefly luciferase luminescence and activation of the *Renilla* luciferase reaction is performed by adding the Stop & Glo® Reagent.

Reactions

1. **ATP + Luciferin** —(**Firefly Luciferase**)→ **AMP + Oxyluciferin + Light (565 nm)**

2. **Coelenterazine + O_2** —(**Renilla Luciferase**)→ **Coelenteramid + CO_2 + Light (480 nm)**

For dual reporter gene assays two injectors are recommended, to guarantee reproducible results and to make the handling more comfortable.
Measurement of the chemical background illustrates the quality of the Assay reagents. The Stop & Glo® Reagent may increase in background due to storage.

Materials

Luminometer:	Orion II Plate Luminometer
Software:	Simplicity 4
Assay :	Dual Luciferase Reporter Assay System
Microplates:	opaque microplates (solid, white, 96 well)

Luminometer and Software are from Berthold Detection Systems GmbH.
The Assay System is supplied by Promega, the microplates by Corning

Method

20µl aliquots of lysates or standards of purified enzymes were put in the wells as duplicates.
Reagents and buffers were prepared as described in the technical manual of the Dual Luciferase Reporter Assay System from Promega.
Injection lines were prepared by priming injector 1 with Luciferase Assay Reagent II (LARII) and injector 2 with Stop & Glo® Reagent. Automatic reagent injector were programmed to dispense 100 µl of each reagent.

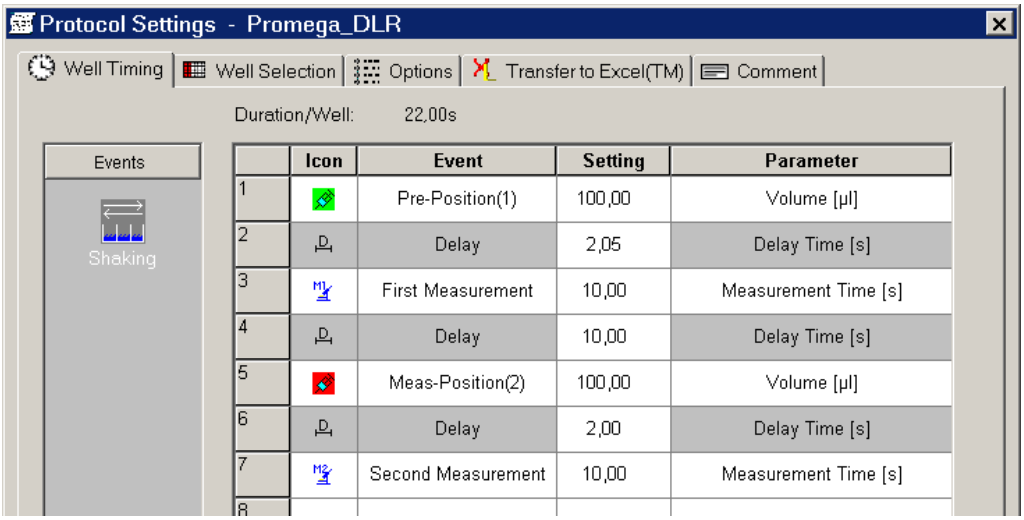
➔ For more details see also the technical manual of the assay .

PC-Settings

- Create a *Dual Measurement* protocol
- Select the microplate format for 96 wells
- Define your well timing (see screenshot below)
- Select the wells you want to measure or choose whole plate
- Select if you want to measure the background, and if you want to save the file automatically
- Activate the automatic Excel transfer and insert the path of the predefined Excel workbook *DLR_Ratio_Simplicity4.xlt* in order to calculate the ratio
- Save the protocol

Event Table in the Dual Measurement Protocol

The screenshot shows the recommended standard settings according to the Promega protocol:



	Icon	Event	Setting	Parameter
1		Pre-Position(1)	100,00	Volume [µl]
2		Delay	2,05	Delay Time [s]
3		First Measurement	10,00	Measurement Time [s]
4		Delay	10,00	Delay Time [s]
5		Meas-Position(2)	100,00	Volume [µl]
6		Delay	2,00	Delay Time [s]
7		Second Measurement	10,00	Measurement Time [s]
8				

Example

Stock solutions of firefly luciferase and *Renilla* luciferase were serially diluted in Passiv Lysis Buffer (PLB) and mixed as described in table 1. Two concentrations of *Renilla* luciferase (sample 1-3 and 4-6) were combined with decreasing firefly luciferase concentrations to simulate different expression level of the reporter enzyme.
Aliquots of 20µl were added in the wells as duplicates. First duplicate is chemical background and contains 20 µl lysis buffer (PLB).

Table of Measured Samples

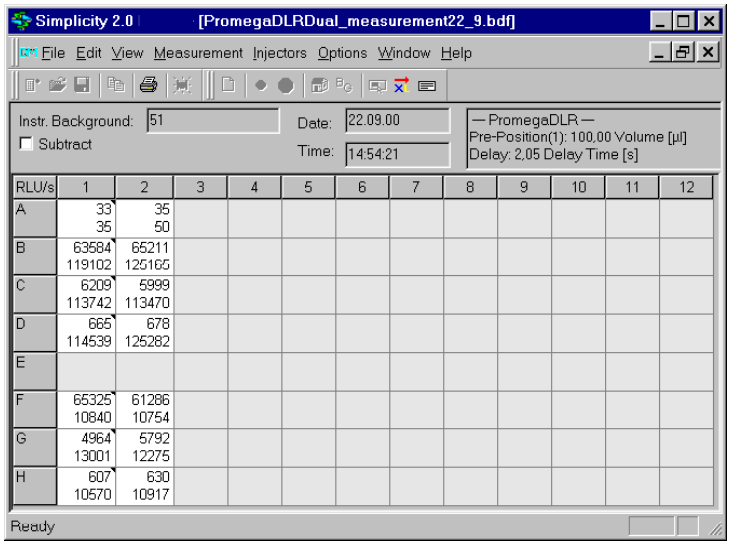
Sample No:	Replicates	dilution firefly luciferase (A)	dilution renilla luciferase (B)	A/B
1	2	10^{-4}	10^{-3}	1
2	2	10^{-5}	10^{-3}	1
3	2	10^{-6}	10^{-3}	1
4	2	10^{-4}	10^{-4}	1
5	2	10^{-5}	10^{-4}	1
6	2	10^{-6}	10^{-4}	1

Microplate Layout

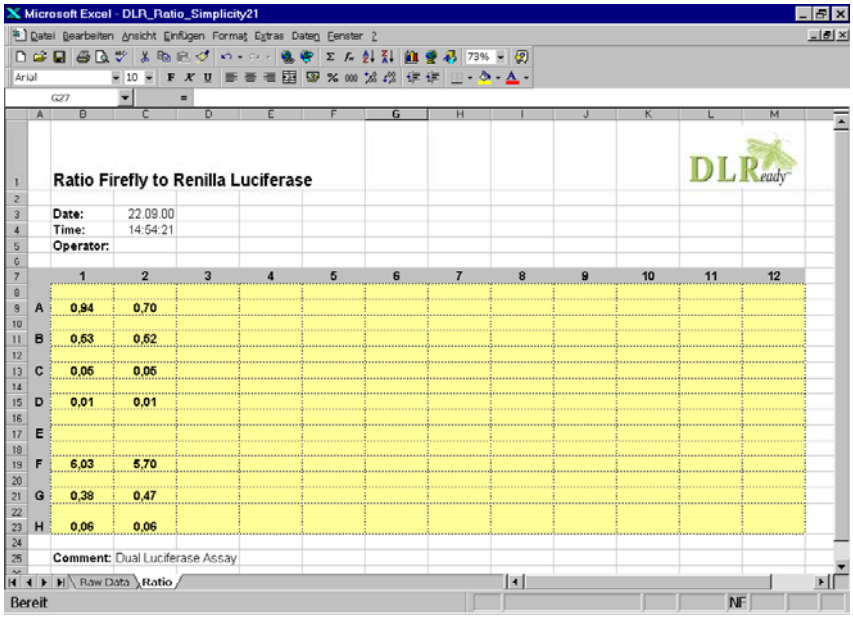
	1	2
A	chemical background	chemical background
B	Sample 1	Sample 1
C	Sample 2	Sample 2
D	Sample 3	Sample 3
E	-	-
F	Sample 4	Sample 4
G	Sample 5	Sample 5
H	Sample 6	Sample 6

Measurement Results

In the measurement window the data are displayed as raw data in RLU/s. Both measurement results are written in one cell of the respective well.



Excel Transfer for ratio calculation



The data will be send to Microsoft® Excel, in the predefined Excel sheet, where the ratio will be calculated automatically.

Discussion:

The measurement simulates two sets of transfection experiments that had been carried out e.g. with two different cell lines. The results of measurement B (Renilla luciferase) illustrate that the control enzyme shows a tenfold higher activity in sample 1-3 than in sample 4-6. The reporter enzyme activity (A) within each set, respective expression in a cell line, covers a range of 3 magnitudes.
In order to find the sample with the highest reporter gene expression, the calculated ratio A / B is analyzed. Whereas the absolute values in light units per second are similar for sample 1 and 4, the normalized result clearly identifies sample 4 with a ratio of about 6 as the sample with the a 12fold higher reporter gene expression than sample 1 (ratio about 0,5).

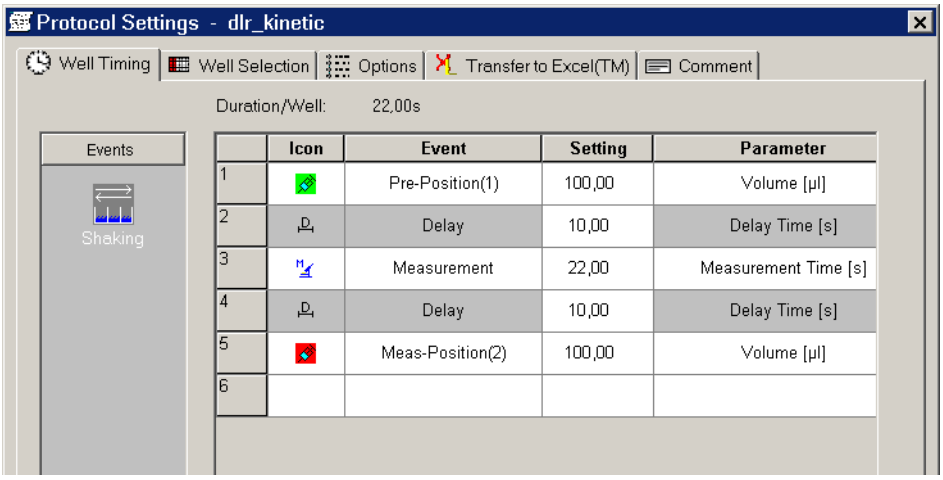
Kinetics

To see if the reagents work well it might be useful to have a look at the kinetic of the reactions. This functionality is provided by the Fast Kinetics Protocol. The light emission is reported over the whole assay time.

PC-Settings

- Create a *Fast Kinetics* protocol
- Select the microplate format for 96 wells
- Define your well timing (see screenshot below)
- Select the wells you want to measure or choose whole plate
- Select if you want to measure the background, and if you want to save the file automatically
- Ignore the automatic Excel Transfer
- Save the protocol

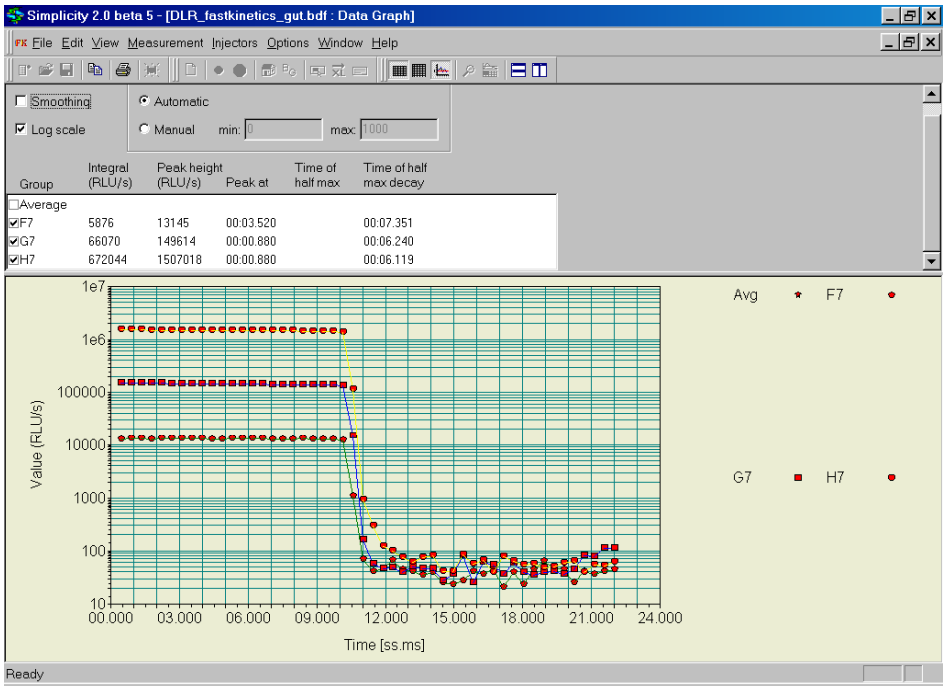
Event Table in the Fast Kinetics Protocol



	Icon	Event	Setting	Parameter
1		Pre-Position(1)	100,00	Volume [µl]
2		Delay	10,00	Delay Time [s]
3		Measurement	22,00	Measurement Time [s]
4		Delay	10,00	Delay Time [s]
5		Meas-Position(2)	100,00	Volume [µl]
6				

The kinetic data of both reactions are collected over a period of 22 seconds.

Graphical Display in the Fast Kinetics Protocol



Reaction A is quenched within 2 sec after addition of the Stop & Glo® Reagent.