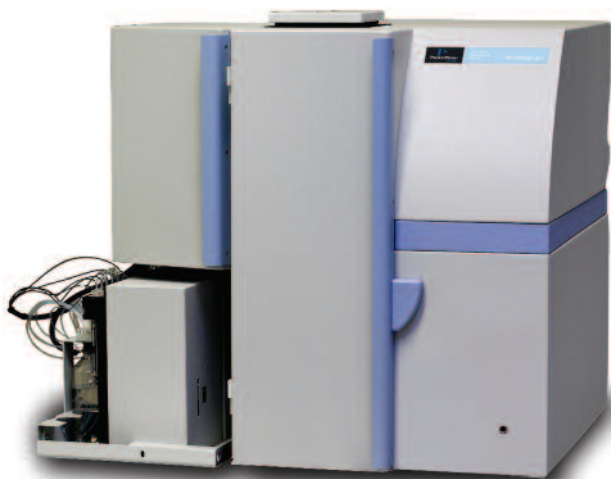


# Dual reporter gene applications using MicroBeta<sup>2</sup> LumiJET



MicroBeta<sup>2</sup> LumiJET

## Introduction

Reporter gene assays are widely used in research of gene activity and protein expression. The study of gene transcription, coupled to the expression of a reporter gene, is a convenient method for quantifying a variety of biological events in cells. Applications of this type include mRNA processing, intracellular signaling, transcription factors and protein folding.

Reporter gene systems typically use enzymes such as chloramphenicol acetyl transferase (CAT),  $\beta$ -galactosidase,  $\beta$ -glucuronidase, firefly luciferase, Renilla luciferase and alkaline phosphatase. Proteins such as growth hormone (hGH) or green fluorescent protein (GFP) are also used.

The Dual-Luciferase<sup>®</sup> Reporter (DLR) Assay System (Promega) contains two different luciferase reporter enzymes that are expressed simultaneously in each cell. Upon a transcriptional activity of the luciferase gene, the functional enzyme is subsequently translated. This translation activity is followed by conversion of a substrate (Luciferine or Coelenterazine) and ATP into visible light. The light output of the reaction is then detected with a luminescence reader.

In dual reporter assays, the “functional” reporter is used to follow a gene of interest while the other reporter serves as an internal “control” for simultaneously tracking a basal response. The control reporter helps to normalize the assay reporter gene results, minimizing

## Author

Miika Talvitie  
 PerkinElmer, Inc.  
 Turku, Finland

variations due to different cell number and transfection efficiency. The firefly luciferase and the Renilla luciferase are sequentially measured from the same sample.

The MicroBeta<sup>®2</sup> Plate Counter is a multi-detector instrument designed for liquid scintillation (LS) counting or luminescence detection of samples in microplates, tubes or on filters. MicroBeta<sup>2</sup> in combination with Promega's DLR assay kit provides an easy, fast and sensitive method for monitoring gene expression. There are two different instrument models available: MicroBeta<sup>2</sup> and MicroBeta<sup>2</sup> LumiJET, which has built-in dispensers. These can have 1, 2, 6 or 12 detectors and can count 24-, 96- and 384-well plate formats. The novel feature in the LumiJET is the inclusion of multiple reagent injectors for measuring "prompt" or "flash" reactions in 384-well plates. Multi-detector LumiJET models can be set to deliver different reagents to the reading position of each detector. MicroBeta<sup>2</sup> LumiJET can be used to dispense two different reagents to each well as required for running dual luciferase assays.

## Materials and Methods

### Materials required

- Promega Dual-Luciferase Reporter<sup>®</sup> Assay System (Promega Cat. No. E1910)
- Firefly luciferase (Promega Cat. No. E1701)
- Renilla reniformis luciferase (Lux Biotechnology Cat. No. 20205)
- MicroBeta2 LumiJET (PerkinElmer 2460)
- White 384-well OptiPlate<sup>™</sup> Microplates (PerkinElmer Cat. No. 60007290)
- Pipettes and Eppendorf<sup>®</sup> tubes

### Preparing the assay reagents

The Dual-Luciferase reporter assay was prepared according to the kit instructions.

Dilution series of the firefly luciferase and Renilla luciferase enzymes were prepared in Eppendorf<sup>®</sup> tubes and kept on ice. A firefly

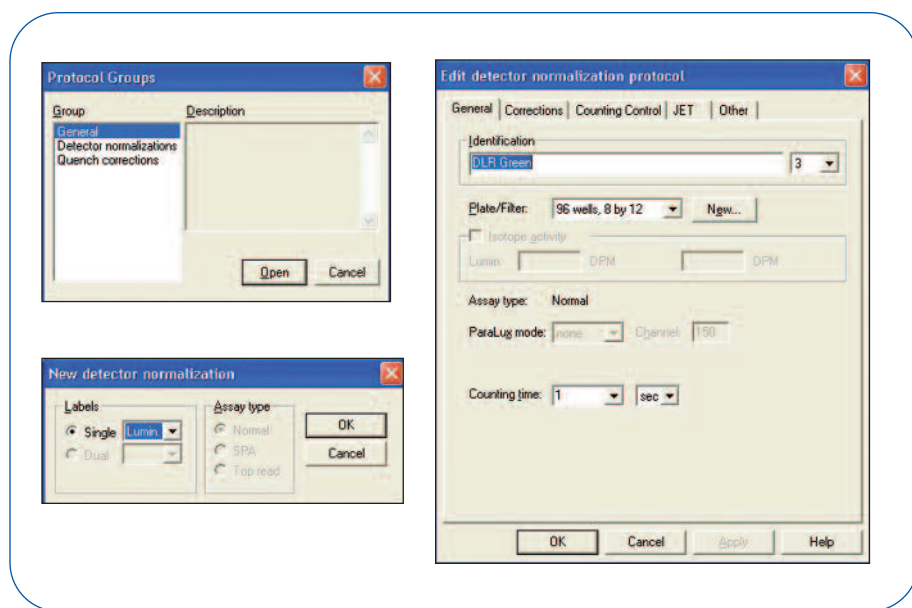


Figure 1. Normalization protocol for DRL green wavelengths (firefly).

luciferase enzyme stock of 13 mg/mL was prepared, using the DLR kit passive lysate buffer (0.1% gelatin). This was further diluted to 8.5E-7 mol/L and 8.5E-8 mol/L (85.2 nM). The Renilla luciferase enzyme stock of 0.25 mg/mL was also prepared using the passive lysate buffer. This was further diluted to 6.9E-5 mol/L and 6.9E-7 mol/L (694.4 nM). Enzyme stocks were then pooled in an Eppendorf tube: 500  $\mu$ L and 61.4  $\mu$ L of firefly and Renilla, respectively ( $2 \times 10^{-12}$  mol/Eppendorf of both enzymes). This concentration was then used to prepare a log dilution series to  $2 \times 10^{-20}$  amol/Eppendorf of both enzymes.

### Preparing the MicroBeta<sup>2</sup> instrument Normalization

Normalization is a necessary step prior to running assays using multi-detector instruments. Normalization is a procedure by which a slight variation between detectors is calculated and correction factors are produced. This ensures that results obtained from any of the detectors are equivalent.

Normalization was done by creating and running two normalization protocols; one for the firefly enzyme (emits green wavelengths) and one for the Renilla

luciferase enzyme (blue wavelengths). Normalization protocols were created by selecting *normalization category* from a protocol list. A *Luminescence* label was selected from a drop-down menu (single). *96-well plate* (general tab) and *use injector module, no dispensing* (JET tab) were activated. Other selections were left as default (Figure 1). As a result, two normalization protocols (DLR green and DLR blue) were created.

Normalization for the DLR assay was done using a fixed luminescence sample emitting green and blue wavelengths. The samples were inserted in two 96-well black OptiPlates (Note: this must be well G11.) The plates were measured with the normalization protocols described above. Normalization always starts with a measurement of an empty black microplate (measurement of detector background values). For this purpose, an empty 96-well black plate was inserted into the instrument first. After the background measurement was complete, the empty plate was replaced with the normalization plate containing a luminescence sample. Measurement was continued by clicking the *next position* button.

## DLR measurement

Two protocols were created, one for a 96-well and one for a 384-well plate (Figure 2).

The *Injector module* was activated from a protocol “JET” tab. *Sample volume* was defined as “20” and “5” for 96-well and 384-well plates, respectively. Both modules (1 and 2) were selected with dispensing volumes of 100 µL and 25 µL for 96-well and 384-well plates, respectively.

Dispensing and measurement were separated with a delay time of 2 seconds (*Dispensing at: -2.0*). For both protocols, a counting time of 10 s and dispensing speed “high” were defined. Note: Any normalization information was attached to the protocols.

A 12-detector MicroBeta<sup>2</sup> LumiJET has 24 injectors. These pumps are divided into two groups both consisting of 12 injectors: “Module1” and “Module2”. Module1 was used to dispense the firefly substrate “LARII” for selected wells. The substrate for Renilla, Stop & Glo<sup>®</sup>, was dispensed using Module2. All the dispensers and tubes were first washed with ethanol followed with water using the *JET dispenser control*.

The DLR kit contains only 10 mL of substrate for both enzymes. For this reason, only pumps 3, 4, 7, 8, 11 and 12 were used. The limited substrate was also taken into account when pipetting enzymes into the plates: only half of a plate (the right side) was used (columns 7 to 12 for 96-well plates and columns 13 to 24 for 384-well plates.) To further save substrate, priming was done only for the tubes required. The tubes of pumps 1, 2, 5, 6, 9 and 10 were left “in air”, outside the substrate bottles.

Plates were prepared by pipetting 20 µL and 5 µL of each dilution (3 replicates) into white 96- and 384-well OptiPlates, respectively (1x10<sup>-21</sup> to 1x10<sup>-13</sup> mol/well of both enzymes). DLR assays were started by putting the plates into the instrument and activating the measurement protocol.

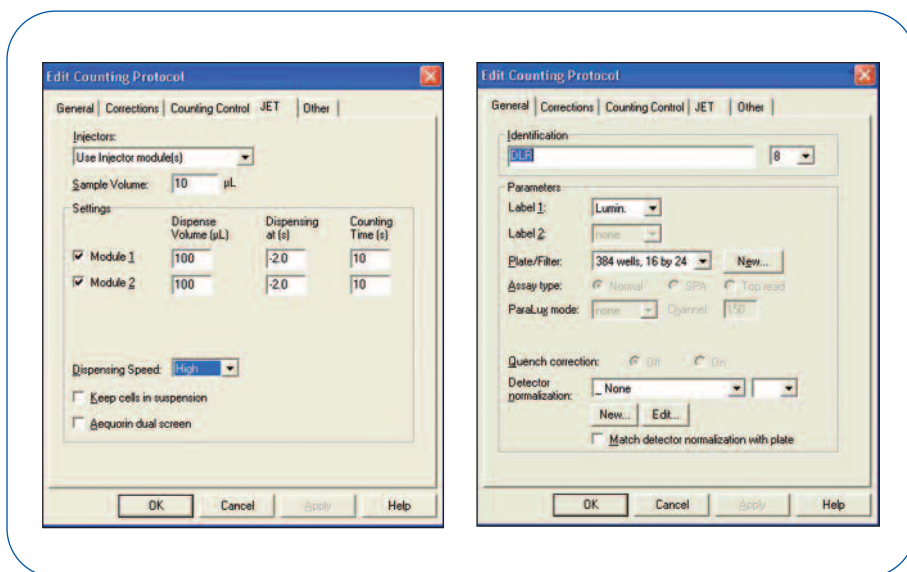


Figure 2. DLR measurement protocol for 384-well plate.

## Analyzing results

*JET DL Ratio assay* software was used to normalize (apply detector normalization information based on the two normalization protocol runs) and calculate ratios of the two enzymes. The following steps were required:

Normalization for firefly: select *file, open* and then select the result file obtained from a DLR green normalization run. Note: Select *Firefly normalization* from the *Files of type* box.

Normalization for Renilla: select *file, open* and select the result file obtained from a

DLR blue normalization run. Note: Select *Renilla normalization* from the *Files of type* box.

Results of DRL run: select *file, open* and select the result file obtained from DLR run. Note: Select *Assay results files* from the *Files of type* box.

LCPS values were copied to MS Excel and followed by Prism<sup>®</sup> (GraphPad Software, Inc.) analysis for standard curves. The lowest detection limits were calculated using the equation:

$$LDL = \text{Average (blank)} + 3SD (\text{blank})$$

		Firefly				Renilla				DLR Blue		
		D	CPS1	CPS1	CCPS1	CCPS1	LCPS	LCPS	LCPS%	LCPS%	Ratio	
1												
2	SAMPLE POS											
3	Unk_1 A07	3	31036878	6669299	31036878	6669299	268944.44	56243.63	0	0	4.618	
4	Unk_1 A08	3	31384395	6672303	31384395	6672303	271937.75	59156.86	0	0	4.597	
5	Unk_1 A09	3	31588498	6733732	31588498	6733731.8	273706.77	59701.50	0	0	4.585	
6	Unk_1 A10	4	4031633	622159	4031633	622159	34349.77	5339.50	0	0.1	6.433	
7	Unk_1 A11	4	4061290	624833	4061290	624833.2	34517.25	5362.45	0	0.1	6.437	
8	Unk_1 A12	4	4064643	631245	4064643	631245.4	34631.02	5417.49	0	0.1	6.392	
9	Unk_1 B07	3	443259	70008	443259	70008.2	3640.73	630.70	0.1	0.2	6.186	
10	Unk_1 B08	3	451513	70006	451513	70006.8	3912.25	630.67	0.1	0.2	6.303	
11	Unk_1 B09	3	442499	69626	442499	69626.2	3834.15	617.31	0.1	0.2	6.211	
12	Unk_1 B10	4	42285	6271	42285	6271.2	360.27	53.82	0.3	0.8	6.694	
13	Unk_1 B11	4	42526	6373	42526	6372.6	362.32	54.69	0.3	0.8	6.625	
14	Unk_1 B12	4	43899	6535	43899	6534.5	374.02	56.08	0.3	0.8	6.669	

Figure 3. JET DL Ratio calculation software for normalization and ratio calculation.

## Results and Discussion

Promega's DLR assay was performed and measured using the MicroBeta<sup>2</sup> LumiJET instrument. Results were normalized, and the ratio of the two enzymes was calculated with *JET DL Ratio* assay software. The software reports raw counts (CPS1), normalized counts (LCPS) and the ratio of Renilla and firefly signals. Sample codes (SAMPLE), wells (POS), detector number (D) are also presented in the software (Figure 3).

Standard curves demonstrate that similar results are obtained on 96-well and 384-well plates (Figures 4 and 5). The assay linear range was 6 orders of magnitude for the firefly enzyme and 5.5 for Renilla with both plate densities. The lowest detection limits, respectively. The corresponding values for 384-well assays were 1.1E-19 and 0.8E-18 mol/well. Performing DLR assays in higher density 384-well plates (384 versus 96) provide increased assay sensitivity (~2-fold) and consumes less (1/4) samples and substrates.

## Summary

The MicroBeta<sup>2</sup> LumiJET with easy-to-use software, built-in dispensers and a maximum of 12 detectors together with 384-well plates provides an extremely robust, sensitive and fast method for running dual reporter gene assays.

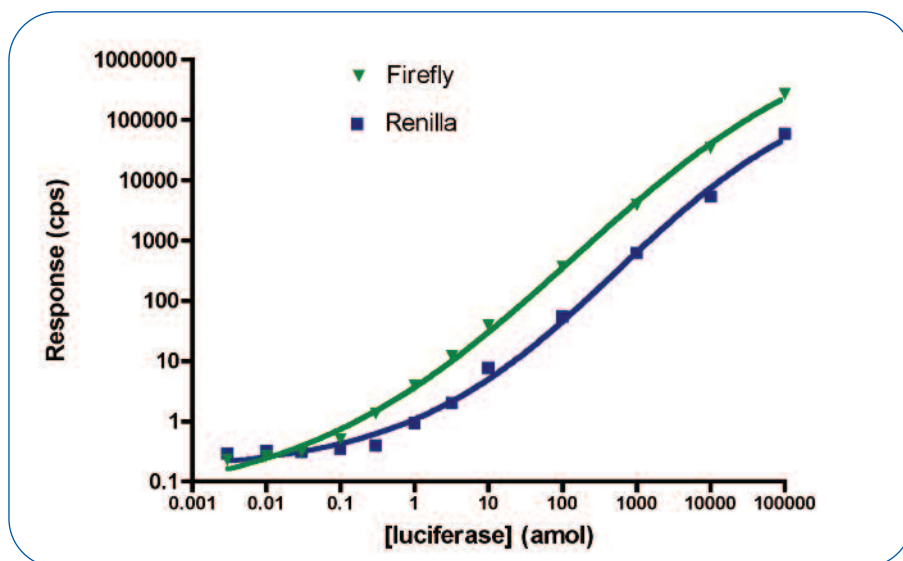


Figure 4. Dilution series of firefly and Renilla luciferase on 96-well plate.

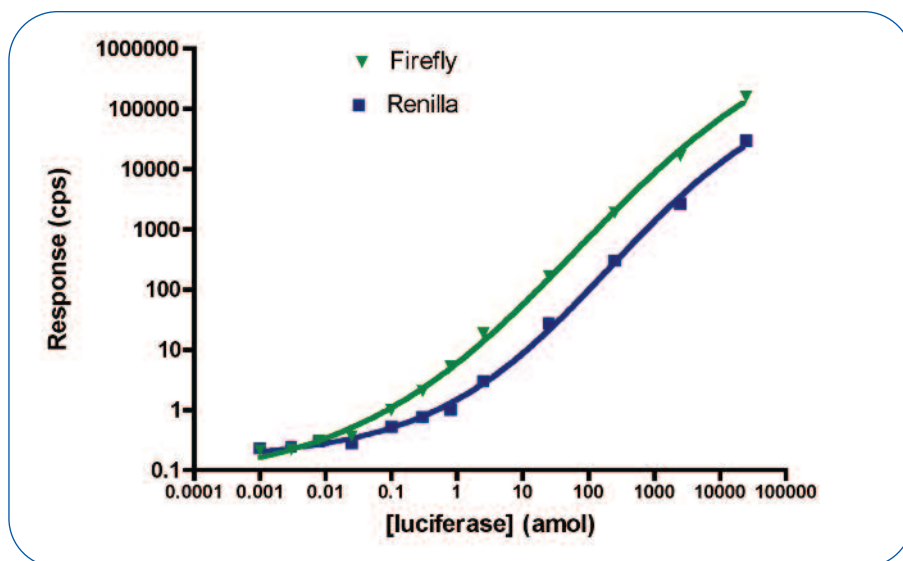


Figure 5. Dilution series of firefly and Renilla luciferase on 384-well plate.