Molecular Devices

Dual-Glo Luciferase Reporter-Gene Assay on the SpectraMax M5, LMax II^{384} and Analyst GT Microplate Readers

SPECTRAMAX APPLICATION NOTE #4

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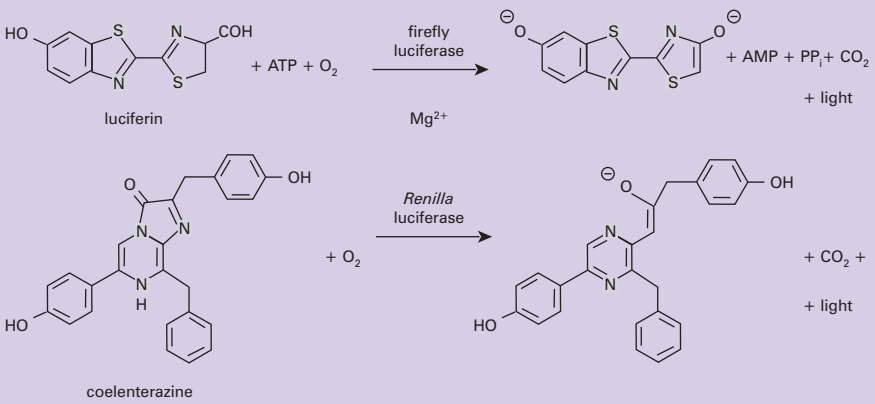
INTRODUCTION

Reporter-gene assays are used to study eukaryotic gene expression. Dual genetic reporters are commonly used in transient transfections of cultured cells to minimize experimental variability caused by differences in cell number, viability or transfection efficiency. One plasmid containing the experimental reporter gene (coupled to a regulated promoter) is cotransfected with a second plasmid containing a control reporter gene (coupled to a constitutive promoter). Bioluminescent reporter systems using firefly and *Renilla* luciferases are widely used as co-reporters because both assays are easy and sensitive. Previously, luciferase reporter assays have been "flash" assays that must be read within seconds of reagent addition and require integrated injectors in the luminometer. (See Maxline App Note #39). Recently, Promega introduced a Dual-Glo Luciferase assay system for high-throughput analyses. The signals are stable for 2 hours after reagent addition and integrated injectors are not necessary.

Reactions Catalyzed by Firefly and *Renilla* Luciferase (Figure 1)



Firefly and *Renilla* luciferase have different substrate requirements.

Figure 1 illustrates the two-enzymatic reactions. Firefly luciferase enzyme catalyzes the oxidation of luciferin with the concomitant release of light.[1] The reaction requires ATP, Mg^{2+} and O_2. *Renilla* luciferase catalyzes the O_2-dependent oxidation of coelenterate luciferin (coelenterazine) but does not require Mg^{2+} or ATP.[2] The enzymes have different substrate requirements, so they can both be measured in a single reaction mixture. Both reactions can easily be measured in Molecular Devices microplate luminometers. Examples of results are presented below for the LMax™ II^{384} dedicated, top-of-the-line microplate luminometer, the Analyst® GT Multi-Mode, Filter-Based Instrument, and the SpectraMax® M5 Multi-Detection, Monochromator-Based Reader with both top- and bottom-read capability.

MATERIALS

- Dual-Glo luciferase assay system (Promega Cat. #E2920)
- Quantilum Recombinant firefly luciferase (Promega Cat. #E1720)
- *Renilla* luciferase (Chemicon Cat. #4400)
- Storage Buffer, 25 mM Tris-acetate, 1 mM EDTA, 1 mM DTT, 0.2 M NH_4SO_4, 15% glycerol, 30% ethylene glycol, 0.1% BSA
- Standard Buffer, Phosphate-buffered saline, pH 7.4 (PBS) plus 0.01% BSA
- Cell line: CHO-K1 (ATCC Cat. #CCL-61)
- Firefly luciferase plasmids: pGL3-control vector (Promega Cat. #E1741) and PCRE-Luc (BD Biosciences Cat. #631911)
- *Renilla* luciferase plasmid: phRL-TK vector (Promega Cat. #E6241)
- Lipofectamine (Invitrogen Cat. #10378-016)
- Opti-Mem (Invitrogen Cat. #31985-070)
- SpectraMax M5 Multi-Detection Microplate Reader, Analyst GT Multimode Microplate Reader and LMax II^{384} Luminescence Microplate Reader (Molecular Devices)

METHODS

Stock standards preparation

Step 1. Stock firefly luciferase standard (1.0 mg/mL) was prepared by adding 0.3 mL of a 14.9 mg/mL stock of firefly luciferase to 4.2 mL of the standard buffer in a plastic vial. The 1.0 mg/mL standard was then divided into 0.5 mL aliquots and stored at -70°C until needed.

Step 2. Daily working firefly luciferase (10 µg/mL) was prepared by transferring 10 µL of the 1.0 mg/mL firefly luciferase to 990 µL standard buffer.

Step 3. *Renilla* luciferase (10 µg /mL) was prepared by adding 990 µL of the standard buffer to the *Renilla* luciferase bottle containing 10 µg of *Renilla* luciferase.

Preparation of combined firefly and Renilla luciferase standards

Step 1. A combined luciferase stock standard (100 ng/mL each) was prepared by transferring 10 µL of each luciferase standard to 980 µL of standard buffer in a plastic vial.

Step 2. The working combined standard curve was prepared by making serial 10-fold dilutions in the standard buffer to achieve 10 ng/mL down to 10 fg/mL each.

Preparation of reagents

Dual-Glo reagent and Dual-Glo Stop & Glo reagent were prepared as directed in the kit instructions.

Assay procedure with luciferase standards

The assay plate was prepared by pipetting 25 µL/well combined firefly/*Renilla* luciferase standards and blanks into a Costar 384-well white plate in replicates of six, followed by 25 µL/well of Dual-Glo luciferase substrate solution to each well. The plate was mixed briefly with a plate shaker, incubated 10 minutes at room temperature in the dark and then read for firefly luciferase activity. According to the kit insert, the firefly luciferase luminescence signal is relatively stable for two hours with greater than 60% of the initial luminescence signal retained in that time.[3] After the firefly luciferase was measured, Dual-Glo Stop & Glo substrate solution (25 µL/well) was added to each well to quench the firefly luciferase reaction and provide substrate for the *Renilla* luciferase reaction. The plate was agitated briefly, incubated 10 minutes at room temperature in the dark and read again. The *Renilla* luciferase luminescence signal is also relatively stable for two hours.[3]

Cell-based assays

CHO-K1 cells were seeded at a density of 2 x 10^6/100 mm plate overnight, and transiently-transfected with pGL3-control vector and phRL-TK vector (6 µg DNA total; pGL3 to phRL-TK ratio = 50:1) following the standard Lipofectamine transfection protocols. The pGL3-control vector contains cDNA-encoding firefly luciferase with expression driven by the SV40 promoter. After 24 hours, the cells were seeded overnight in 25 µL at densities of 0 to 25,000 cells/well in a Costar white wall/clear bottom 384-well plate (n=6/group). The following day, Dual-Glo luciferase substrate solution was added (25 µL/well). The plate was agitated briefly and incubated 10 minutes at room temperature in the dark to ensure complete cell lysis and enzyme equilibration. It was then read for firefly luciferase activity. After the firefly luciferase was measured, Dual-Glo Stop & Glo substrate solution (25 µL/well) was added to each well to quench the firefly luciferase reaction and provide substrate for the *Renilla* luciferase reaction. The plate was agitated briefly, incubated 10 minutes at room temperature in the dark and read again.

To measure induction of pCRE-luc (cAMP response element) by Forskolin and 8-Br-cAMP, CHO-K1 cells were seeded overnight at a density of 2 x 10^6/100 mm plate and transiently-transfected with pCRE-luc and phRL-TK vectors (6 µg DNA total; pCRE-luc to phRL-TK ratio = 50:1) following the standard Lipofectamine transfection protocols. 24 hours after transfection, cells were seeded overnight in 100 µL (60,000 cells/well) in a Costar white wall /clear bottom 96-well plate, then treated with 50 µM Forskolin or 1 mM 8-Br-cAMP for 6 hours (Control = untreated). Firefly (pCRE-Luc) and *Renilla* (phRL-TK) luciferase activities were measured with the Dual-Glo assay system.

Instrument settings

SpectraMax M5 Microplate Reader:

Read type = endpoint, Read Mode = luminescence with 1000 ms integration time, Wavelengths = All, Automix = off, Auto calibration = on, Settling time = off, Autoread = off.

LMax II^{384} Microplate Luminometer:

Integration time = 1 second, Settling time = off, Autoread = off.

Analyst GT Microplate Reader:

Luminescence height = 1 mm, max integration as 1,000,000 µsec., target CV per well = 1.0%, raw data units = counts/sec., attenuation mode = out.

Data analysis

Molecular Devices SoftMax® Pro software was used to analyze and present the data. The lower limit of detection (LLD) was defined as the level of luciferase detectable above 3 positive standard deviations of the blank.

RESULTS

Luciferase emission spectrum

Figure 2 shows the emission spectrum of firefly luciferase obtained on the SpectraMax M5. The emission lambda maximum was 560 nm, as has been reported in the literature. The emission lambda max of *Renilla* luciferase is approximately 480 nm (data not shown).

Luciferase standard curves

Figure 3 shows representative firefly and *Renilla* luciferase standard curves obtained in a 384-well plate in the LMax II^{384}. The firefly luciferase signal was an order of magnitude greater than that of the *Renilla*. In this particular experiment, the estimated lower limits of detection (LLDs) were 2 and 20 fg/well for firefly and *Renilla* luciferase, respectively in both the LMax II^{384} and the Analyst GT Systems .

Figure 4 shows a firefly luciferase dilution series measured in a 96-well plate in the SpectraMax M5 and the Analyst GT. Typical LLDs were approximately 1–2 fg/well in the Analyst GT and 2–3 fg/well in the SpectraMax M5. The dynamic range of the assay was approximately 5 orders of magnitude.

Cell-based experiments

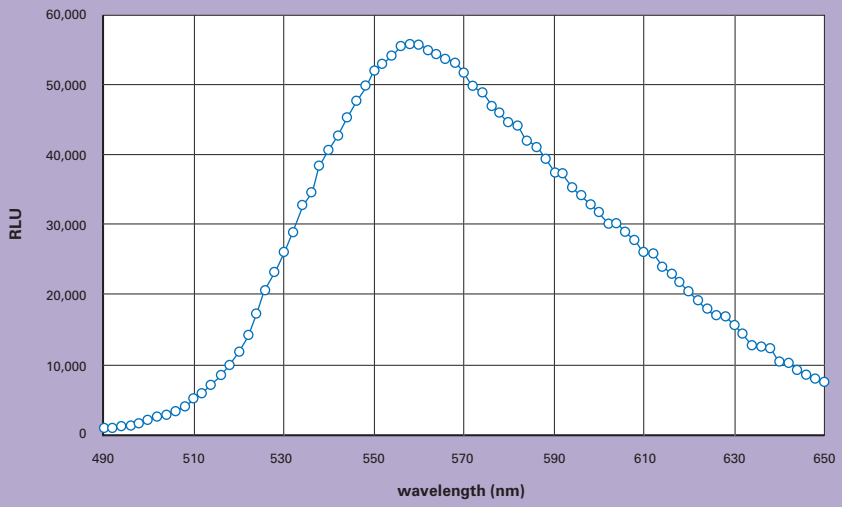
In the first experiment, we prepared a dilution series of CHO-K1 cells transiently transfected with pGL3-control vector and phRL-TK vector. The cells were seeded overnight at densities varying from 0 to 25,000 cells/well in a 384-well plate and firefly and *Renilla* luciferase activities were measured in an LMax II^{384} (Figure 5) and an Analyst GT (data not shown). The estimated LLD was 25 cells/well in firefly luciferase activity in both instruments and the Z' factors of LLD were similar (0.52 in LMax II and 0.58 in Analyst GT). SpectraMax M5 would give similar results, although these particular experiments were done before the SpectraMax M5 became available.

Note: the Dual-Glo reagent lyses the cells and releases the enzymes into the solution, so the luminescence measurement is basically the same as with purified enzymes in solution. The Spectramax M5 has similar detection capability to the Analyst GT and LMax II^{384} (Figure 4), so we are confident that its performance is also similar when measuring the enzymes in cell lysates.

The purpose of the next experiment was to demonstrate that the Dual-Glo assay system could be used to measure the induction of pCRE-luc by forskolin and 8-Br-cAMP. Figure 6, Panel A shows the firefly and *Renilla* luciferase activities measured with the Dual-Glo assay system in an LMax II^{384}. The marked induction of firefly luciferase activity by forskolin and 8-Br-cAMP is obvious. As expected, there is little or no effect on *Renilla* activity. Similar results were obtained on the Analyst GT.

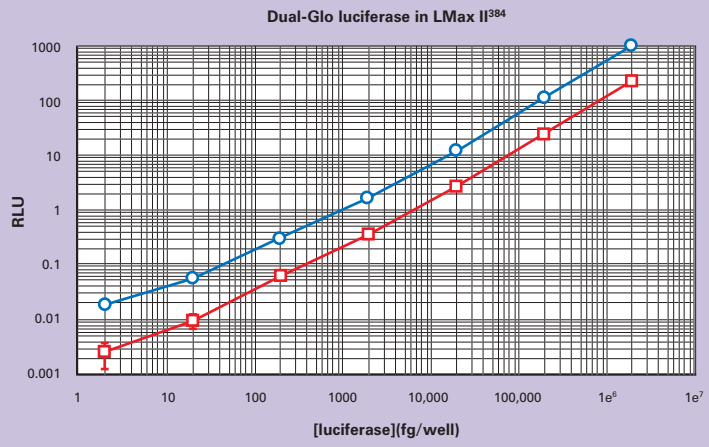
Figure 6, Panel B shows the same results, after the firefly activity has been normalized to *Renilla*

firefly luciferase emission spectrum (figure 2)



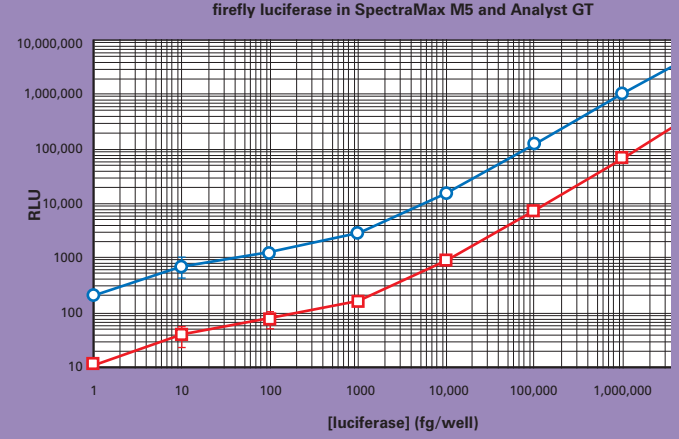
Firefly luciferase emission spectrum (10 ng enzyme /mL) obtained in a SpectraMax M5 multi-detection microplate reader.

luciferase standard curves (figure 3)



Firefly (circles) and *Renilla* (squares) Luciferase Standard Curves obtained in the LMax II^{384}. Similar curves were obtained in Analyst GT.

firefly luciferase dilution series (figure 4)



Firefly luciferase dilution series read in the Analyst GT (circles) and SpectraMax M5 (squares) readers.

activity (*i.e.,* ratio of firefly activity to *Renilla* activity). The induction of firefly activity is approximately 3-fold and the Z' factors for both instruments were 0.64 and 0.62 for forskolin at 50 µM and 8-Br-cAMP at 1 mM respectively.

DISCUSSION AND CONCLUSIONS

The results above revealed excellent detection limits and dynamic range in all three Molecular Devices microplate luminometers. The estimated lower limit of detection (LLD) in both 96- and 384-well plates is < 2 fg/well for firefly luciferase in the LMax II^{384} and Analyst GT and 2–3 fg/well in the SpectraMax M5. *Renilla* luciferase emission is much dimmer than the firefly and thus its LLD is 10 times higher.

The LLD for firefly luciferase activity in transiently-transfected CHO-pGL3-luc-phRL-TK cells was 25 cells/well (Z' factor = 0.58 and 0.52 for LMax II^{384} and Analyst GT respectively). Results would be similar for the SpectraMax M5, based on results of several experiments with purified enzymes (*e.g.,* Figure 4). As pointed out above, once the Dual-Glo reagent is added, the cells become lysed and the enzymes are free in solution during the actual luminescence measurements, just as they are with the enzyme standard preparations.

The instruments can also easily detect Forskolin and 8-Br-cAMP induced pCRE-luc activity in transiently transfected CHO-pCRE-luc-phRL-TK cells (Z' factors were 0.64 and 0.62 for forskolin and 8-Br-cAMP respectively).

The LMax II^{384} is a dedicated, top-of-the-line microplate luminometer. It provides the sensitivity, flexibility, automation capability and validation tools required by leading laboratories and is suitable for high-throughput applications. The LMax II^{384} with dual liquid injectors is designed for both flash and glow luminescence detection applications. Ultra-fast photon counting technology provides superior signal-to-noise ratio. The LMax II^{384} can be integrated with robotic systems.

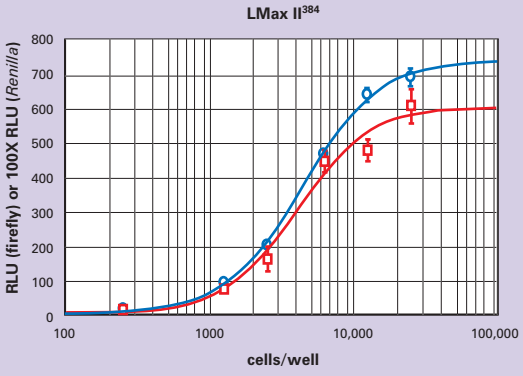
The Analyst GT delivers unsurpassed multimode assay detection performance including an independent optical system and photomultiplier tube for luminescence detection. The dedicated luminescence PMT has extremely low background counts and superior performance in the blue wavelengths. The Analyst GT is equipped with 20 plate magazines in a bi-directional stacker.

The SpectraMax M5 is a multi-detection, monochromator-based reader with both top- and bottom-read capability. It offers wavelength scanning capability, as well as luminescence performance competitive with dedicated luminometers.

REFERENCES

1. M.A. DeLuca and W.D. McElroy. (1978) in *Meth Enzymol* Vol. 53; p. 3.
2. J.C. Matthews, *et al.* (1977) Purification and properties of *Renilla* reniformis luciferase. *Biochemistry*, 16:58.
3. Promega, Dual-Glo luciferase assay system technical manual, www.promega.com.

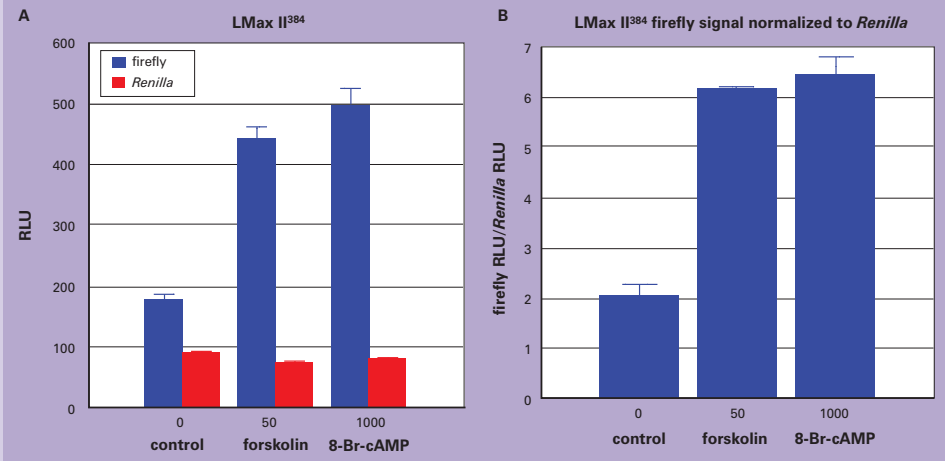
cell-based standard curve (figure 5)



y = ((A-D)/1 + (x/C)^B)) = + D	A	B	C	D	R^2
firefly (pGL3: conc. vs. mean value)	9.106	1.646	4452.807	741.612	0.999
Renilla * 100 (phRL-TK)	8.581	1.741	4091.141	607.002	0.998

Dual-luciferase activity as a function of cell number in the LMax II^{384}. Firefly activity is shown in blue (circles) and *Renilla* activity in red (squares). In order to display both plots on a single graph, the *Renilla* activity is scaled by 100.

induction of firefly luciferase (figure 6)



A: Measurement of induction of pCRE-luc by forskolin and 8-Br-cAMP in the LMax II^{384}. Firefly (pCRE-Luc) and *Renilla* (phRL-TK) luciferase activities were measured with the Dual-Glo assay system. B: Measurement of induction of pCRE-luc by forskolin and 8-Br-cAMP in the LMax II^{384} (firefly activity normalized to *Renilla*).

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