Dual Luciferase® Reporter Assay Detection on Next-Generation FlexStation® 3 Microplate Reader with Integrated Fluidics

Carole Crittenden and Cathy Olsen, PhD.
Molecular Devices Corporation, Sunnyvale, CA

Introduction

Reporter-gene assays are useful tools in the study of eukaryotic gene expression. Dual-Luciferase Reporter Assay System from Promega utilizes differing luminescent properties of firefly and *Renilla* luciferase to normalize activity of an experimental reporter with an internal control. FlexStation 3, a next-generation multi-mode microplate reader, with liquid transfer capabilities provides a robust platform for increased throughput of this important flash luminescence assay. Built on the proven SpectraMax® M5e optical platform, FlexStation 3 expands capacity to perform real time fast kinetic assays by pipetting and reading simultaneously.

Firefly and *Renilla* Luciferase enzyme standards are diluted in Passive Lysis Buffer (PLB). Firefly luciferase luminescence is initiated by addition of Luciferase Assay Reagent II (LAR II). Addition of Stop & Glo® Reagent simultaneously quenches firefly luciferase signal and initiates Renilla luciferase luminescence. Curves are generated in SoftMax® Pro software.

Dual Luciferase Assay Principle

The firefly and renilla luciferase reactions are shown below. The firefly enzyme catalyzes the ATP-, Mg^{2+} and O_2–dependent oxidation of luciferin with the concomitant release of light. Renilla luciferase catalyzes the O_2-dependent oxidation of coelenterate luciferin (coelenterazine) but does not require Mg^{2+} or ATP.

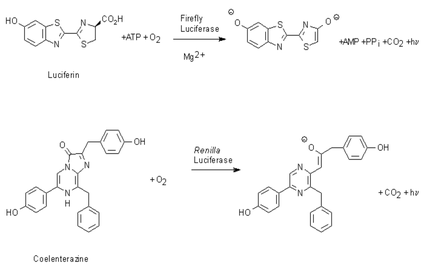


Figure 1. Chemistry of luciferase enzyme assay Diagram courtesy of Promega corp.

Benefits of Integrated Fluidics

Easily changed 8- and16-channel pipette heads enable assays in 96- and 384-well plates. Multiple additions can be made to the same well enabling agonist and antagonist assays. Assays are easily optimized with user defined height, speed of addition, reagent source, and tip column for each addition. Less dead volume saves precious reagents compared to injectors. Disposable pipette tips reduce opportunity for cross-contamination between additions and experiments. Triturating in the well after addition facilitates mixing in low volume assays.

FlexStation 3 Flex Mode Dual-Luciferase Standards Assay

Materials

Dual-Luciferase® Reporter Assay System kit (Cat# E1960, Promega Corporation) including Passive lysis buffer(PLB) 5X reagent, Luciferase assay buffer(LAR II), Luciferase assay substrate, Stop & Glo® Substrate 50X and Stop&Glo® buffer.
1 mg/mL Firefly Luciferase (Cat# 9506, Sigma) in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and frozen in aliquots at -70° C until use. When needed, a vial is thawed and a 10 μg/mL standard prepared by diluting 10 μL stock with 990 μL PLB.
10 μg/mL stock recombinant Renilla luciferase (Cat# 4400, Novalite, Chemicon International) made by reconstituting a 10 μg vial in 990 μL PLB.
White-wall, clear bottom 96- and 384-well TC-treated polystyrene plates (Cat# 3604 and 3707 respectively, Corning)

Methods

Prepare 1X passive lysis buffer by diluting 5X PLB with deionized water. Dual luciferase working standard is prepared by diluting 10 μL each of 10 μg/mL firefly and 10 μg/mL *Renilla* luciferase with 980 μL PLB (concentration of each enzyme = 100 ng/mL). Make serial half-log dilutions in PLB. Diluted firefly+*Renilla* luciferase standards are pipetted into microplate wells in quadruplicate. 96-well assay plates are prepared by pipetting 20 μL/well. 384-well plates are prepared by pipetting 5 μL/well.
Prepare LAR II reagent and Stop & Glo reagent following Promega kit instructions. Aliquot enough of each reagent into appropriate 96- or 384-well polypropylene plates to complete the assay.
Using Flex Mode, set up and run assay protocol to simultaneously pipette LAR II and detect luminescence. Using a second addition step, continue reading while adding Stop & Glo to quench firefly luciferase luminescence and initiate *Renilla* luciferase luminescence. FlexStation 3 Flex Mode protocol details are listed in Table 1.

Table 1. FlexStation 3 Luciferase Assay Set-up Parameters

Parameter	Setting
Read mode	FLEX
Read type	Luminescence, bottom read
Wavelengths	All
Timing	90 seconds total
Autocalibrate	Once
Assay plate type	384-well white/clear bottom plate
Compound source	384-well polypropylene plate
1st Compound transfer	Add 25 μL, LARII at 20 μL pipette height, addition rate 7, addition time 21 sec.
2nd Compound transfer	Add 25 μL Stop & Glow at 25 μL pipette height, addition rate 7, addition time 54 sec.
Trituration	Triturate after the 2nd addition step. Height 30 μL, volume 25 μL
Compound and tip columns	Varies according to plate layout
Auto read	Off

96-well plates volumes are 4X those of 384-well plates

96-Well Dual-Luciferase Assay on FlexStation 3

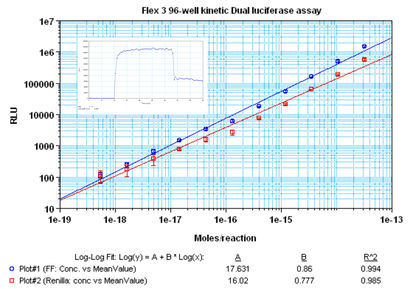


Figure 2. Dual-Luciferase assay performed in a 96-well white walled, clear bottom plate. In Flex Mode, FlexStation 3 microplate reader was set to read luminescence from the bottom of the plate. During the kinetic read, 2 additions were made. RLU values were calculated as area under the curve for 10 seconds after addition of each reagent. Lower Limit of Detection (LLD) is calculated as the concentration between two datapoints on the linear part of the curve equal to 3*SD+background. Firefly luciferase LOD= 0.77 attomole, R^2= 0.994, and *Renilla* luciferase LLD= 1.3 attomole, R^2= 0.985.

384-Well Dual-Luciferase Assays on FlexStation 3

Trituration after addition

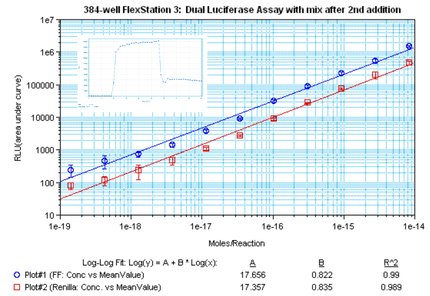


Figure 3a. In 384-well mode, a trituration step was added after pipetting each reagent increasing linearity at lower concentrations of luciferase. Firefly luciferase LLD= 0.72 attomoles, R^2= 0.99 and *Renilla* luciferase LLD= 0.57 attomoles, R^2= 0.989 .

No trituration after addition

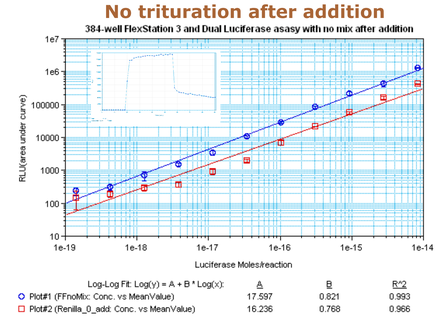


Figure 3b. In 384-well mode, no trituration step was added after pipetting each reagent. Firefly luciferase LLD= 0.69 attomoles, R^2= 0.993, and *Renilla* luciferase LLD= 40 attomoles, R^2= 0.966. Linearity of Renilla luciferase was affected at lower concentrations due to lack of mixing.

SpectraMax M5e platform Dual-Glo® Luciferase Cell Standards Assay

CHO-K1 cells were seeded at a density of 2 x 10^6 cells per 100-mm plate overnight, then 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 50,000 cells/well in a white wall/clear bottom 384-well plate (n = 6/group). The following day, Dual-Glo luciferase substrate solution was added offline (25 μL/well). The plate was agitated briefly and incubated 15 minutes at room temperature in the dark to ensure complete cell lysis and enzyme equilibration. It was then read for firefly luciferase activity on SpectraMax M5e. After the firefly luciferase was measured, Dual-Glo Stop & Glo substrate solution (25 μL/well) was added offline to each well to quench the firefly luciferase reaction and provide substrate for the *Renilla* luciferase reaction. The plate was agitated briefly, incubated 15 minutes at room temperature in the dark and read on M5e again.

Dual-Glo Luciferase Cell Titration

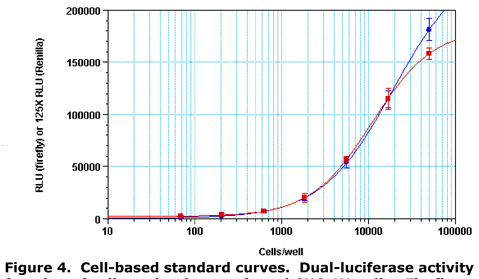
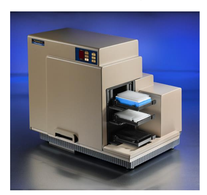


Figure 4. Cell-based standard curves. Dual-luciferase activity as a function of cell number in transfected CHO-K1 cells. Firefly activity is shown in blue (circles), and *Renilla* activity is shown in red (squares). In order to display both plots on a single graph, the *Renilla* activity is scaled by 125. Data shown was read using SpectraMax M5e which shares its optical platform with FlexStation 3.

Conclusions

Utilization of FlexStation 3 pipetting mode to add reagents and triturate during reading improves Dual-Luciferase enzyme standards assay results at lower concentrations in 384-well plates. Based on promising Dual-Glo cell data on Spectamax M5e, future experiments are planned to investigate the utility of FlexStation 3 fluidics to enable flash Dual-Luciferase cell based assays in 384-well format.



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