SENSITIVE DETECTION OF REPORTER GENE ASSAY RESULTS

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The number of primary cortical cells and neuronal populations from cell lines becomes limiting when multiple treatments or conditions are tested. Therefore assays and instrumentation used to study gene expression in neuronal cell types need to be sensitive enough to work with limited cell numbers. Here we describe and evaluate a reporter gene assay and instrumentation for use in such cell populations.

Introduction

The ability to introduce and express DNA in neuronal and glial cells is essential for understanding the function of the thousands of genes expressed in the brain. During the last two to three decades, several gene transfer methods have been developed and tested including viral vectors, liposomes and electroporation (reviewed in reference 1). However, each transfection method has its own merits and limitations depending upon cell type and experimental conditions (1–3). Poor transfection and variation in transfection efficiency among treatment groups may directly affect assay sensitivity and experimental results for studies of gene expression using genetic reporters.

Here we describe a new reporter gene assay for neuronal and glial cells and evaluate instrumentation sensitivity. We evaluate these factors using several cell lines transfected with the pGL3-Basic Vector[a,b] using the Dual-Glo™ Luciferase Assay System[a,c-g] and three different luminometers.

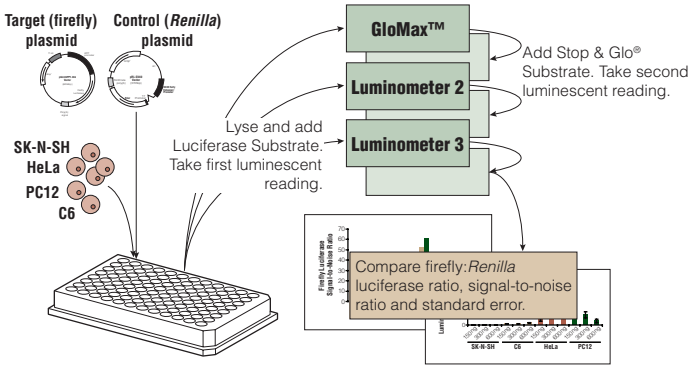

Figure 1. Flow chart of instrumentation comparison experiment. Cells were seeded in 96-well plates, allowed to attach overnight, then transfected with an experimental and a control plasmid. Twenty-four hours later, buffer containing firefly luciferase substrate was added and four readings were taken in each of the three instruments. Stop & Glo® Reagent was then added to stop the firefly reaction and measure *Renilla* luciferase activity, and four more readings were taken with each instrument. The main endpoints for instrument comparison were standard error of the four measurements and signal-to-noise ratio. In addition, firefly/*Renilla* luciferase ratio allowed us to compare promoter fragment activity among cell types.

Materials and Methods

Experimental Overview. Commonly used cell lines, SK-N-SH (human neuroblastoma), C6 (mouse glioma), PC12 (rat pheochromocytoma), and HeLa (human cervical cancer cells) were obtained from ATCC. Transfection efficiencies of the cell lines were compared using varying amounts of DNA at differing cell densities in the culture plate. In addition to the pGL3 firefly luciferase reporter vector, pRL-SV40 Control Vector expressing *Renilla* luciferase was cotransfected into the cells, and both luciferase activities were measured and checked for cross-talk using three different luminometers: the GloMax™ 96 Microplate Luminometer, Luminometer 2 and Luminometer 3. For each instrument, variability between repeated measurements of the same samples is reported as coefficient of variation (CV); signal-to-noise ratios are also reported (Figure 1).

Luciferase Reporter Constructs. A 1,235bp 5′-flanking region of the *Rhesus* amyloid precursor protein (APP) gene (4), spanning –1,131 to +104 of the transcription start site, was inserted into the pGL3-Basic Vector (Cat.# E1751) upstream of the firefly luciferase reporter gene (Figure 2). We previously checked the promoter activity of this fragment with different reporter systems and found that it was moderately active in several cell lines (5). The pRL-SV40 plasmid containing the *Renilla* luciferase gene under a highly active constitutive promoter was used as an internal control to determine transfection efficiency.

Luminescence Detection Instrumentation. To compare luminometers, SK-N-SH cells were seeded at a density of 40,000 cells/well in a 96-well plate and allowed to attach overnight. These cells were then transfected with the pGL3-APP plasmid and cotransfected with the *Renilla* luciferase vector. Twenty-four hours after transfection, the cells were assayed for firefly and *Renilla* luciferase activities using the Dual-Glo™ Luciferase Assay System (Cat.# E2920). After a 10-minute incubation with the luciferase detection reagents, four readings were taken in rapid succession (at approximately 1-minute intervals) in each of the three luminometers to assess instrument variability (Figure 4). The Dual-Glo™ signal is sustained over at least two hours, so reduction of signal is negligible over the time required to take the measurements described. Several controls were included

Sensitive Detection of Reporter Gene Assay Results

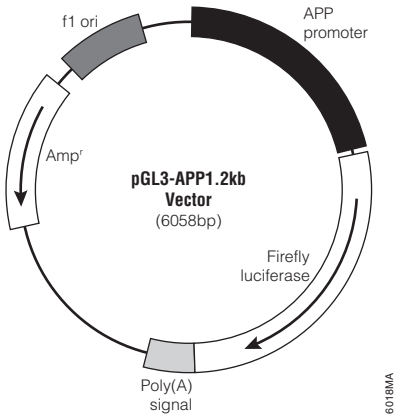

Figure 2. For these experiments, 1,235bp of the *Rhesus* amyloid precursor protein (APP) promoter was cloned upstream of firefly luciferase in the pGL3-Basic Vector.

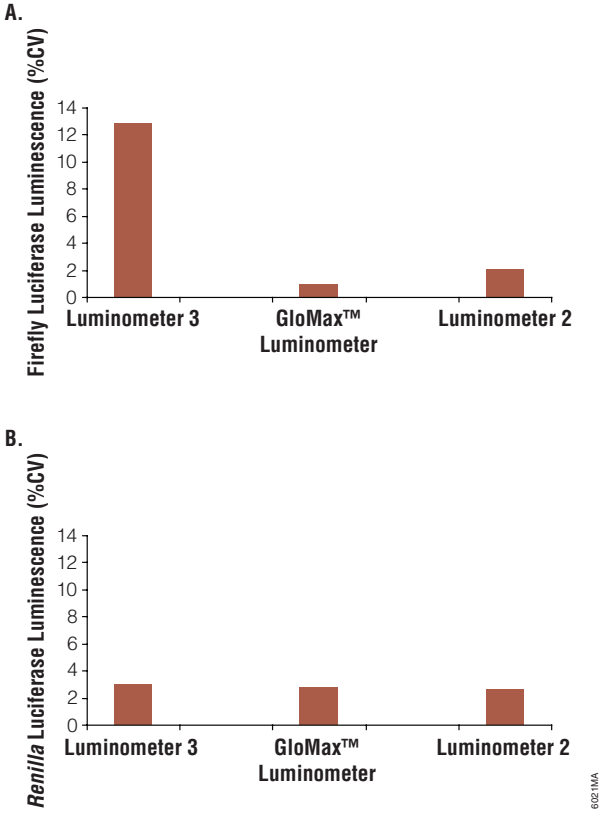

Figure 3. Coefficients of variation for three luminometers. Panel A shows highest variability in Luminometer 3 and lowest in the GloMax™ Luminometer. **Panel B** shows nearly equal performance of the three instruments with *Renilla* luciferase. Absolute luminescence of *Renilla* was approximately 36-fold higher on average, so these instruments produce data of roughly equivalent quality with very strong signals.

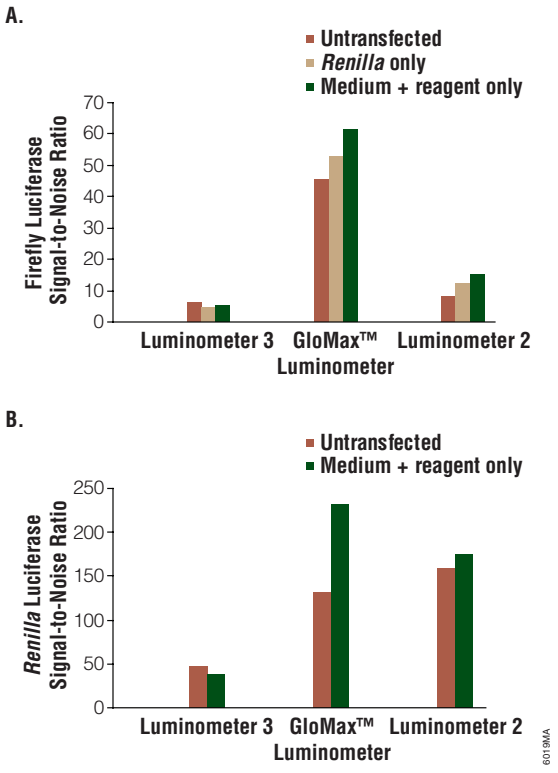

Figure 4. Signal-to-noise ratio. Panel A shows that the highest signal-to-noise ratio in the firefly luciferase assay was achieved with the GloMax™ Luminometer, while Luminometers 2 and 3 performed poorly in comparison. Luminescence was compared between transfected cells and three different measures of background: untransfected cells, cells transfected with *Renilla* luciferase only, and media with detection reagents but no cells. *Renilla* luciferase produced no detectable luminescence with firefly luciferase detection reagent. **Panel B** shows more equivalent performance between Luminometer 2 and the GloMax™ Luminometer but poor performance from Luminometer 3 in the *Renilla* luciferase assay.

to determine signal-to-noise ratios: medium-only, untransfected cells, and cells transfected only with the *Renilla* luciferase vector.

Comparing Detection Sensitivity of Three Luminometers

Each instrument produces raw data in different absolute ranges, so a coefficient of variation (CV) was calculated over the four readings of both firefly and *Renilla* luciferase signals. The CV for Luminometer 3 was much higher than that for the GloMax™ Luminometer or Luminometer 2 for the firefly signal, but all instruments had comparable CVs for the *Renilla* luciferase measurements (Figure 3). The difference in CVs for the two luciferase signals is probably a result of the severalfold higher activity observed for the *Renilla* luciferase internal control.

Signal-to-noise ratios were calculated for both firefly and *Renilla* luciferase assays using several different control conditions as "noise" (Figure 4). For the firefly luciferase signal measurements, the signal-to-noise ratios for Luminometers 2 and 3 were comparable but much lower than those for the GloMax™ Luminometer. For the *Renilla* luciferase measurements, the GloMax™ Luminometer and Luminometer 2 both outperformed Luminometer 3 (Figure 4). Activity of the *Renilla* luciferase reporter was 36-fold higher on average than the firefly luciferase activity under the APP promoter, which may explain disparities between the abilities of the instruments to measure the two different luciferase signals.

Summary

In our comparison of instruments, the GloMax™ Luminometer and Luminometer 2 outperformed Luminometer 3 in measures of both within-assay variability and signal-to-noise ratio. The GloMax™ Luminometer and Luminometer 2 were generally equivalent in the quality of data produced when measuring the signal from the *Renilla* control; however, the signal-to-noise ratio heavily favors the GloMax™ instrument when measuring firefly luciferase signal under the control of the APP promoter. The disparity between the signal-to-noise ratios for firefly and *Renilla* luciferase measurements can be explained by a difference in the raw data. The *Renilla* luciferase vector contained a strong promoter and, as a result, produced a manyfold higher raw signal. However, the less active APP promoter fragment is more typical of experimental conditions commonly encountered in the laboratory, and in this study the GloMax™ Luminometer performed the best of the three luminometers tested.

We conclude that the quality of data produced by the GloMax™ Luminometer and Luminometer 2 is generally better than that of Luminometer 3 and that the GloMax™ Luminometer is the best of the three for low signal intensities. For ease of handling, Luminometer 3 and the GloMax™ Luminometer are superior because all functions are controlled directly through an Excel macro and all data are recorded into Excel spreadsheets. Additionally, downloadable, pre-optimized software settings for the Promega luminescent assays are available for the GloMax™ Luminometer. Although less user-friendly, Luminometer 2 software provides a wider variety of functions including higher level statistical analysis, and data can be exported to a variety of file formats, though it lacks the convenience of recording data directly into a spreadsheet. Luminometer 3 is a good, multifunctional instrument that should only be used in luminescence assays with strong signals. For all considerations mentioned here, the GloMax™ Luminometer appears to be the superior instrument of the three. The conditions used here allowed us to detect the luciferase reporter gene driven by a moderately active promoter. Notably, this can be achieved from as low as 150ng DNA in 25,000 cells 24 hours after transfection in a high-throughput system.

References

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Acknowledgment

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Protocols

Dual-Glo™ Luciferase Assay System Technical Manual #TM058 (www.promega.com/tbs/tm058/tm058.html)

GloMax™ 96 Microplate Luminometer Technical Manual #TM278 (www.promega.com/tbs/tm278/tm278.html)

pGL3 Luciferase Reporter Vectors Technical Manual #TM033 (www.promega.com/tbs/tm033/tm033.html)

Ordering Information

Product	Size	Cat.#
pGL3-Basic Vector	20µg	E1751
GloMax™ 96 Microplate Luminometer	1 each	E6501
Dual-Glo™ Luciferase Assay System	10ml	E2920
pRL-SV40 Vector	20µg	E2231

[a]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673. A license (from Promega for research reagent products and from The Regents of the University of California for all other fields) is needed for any commercial sale of nucleic acid contained within or derived from this product.

[b]U.S. Pat. No. 5,670,356.

[c]U.S. Pat. Nos. 7,078,181, 7,108,996, 7,118,878 and other patents pending.

[d]U.S. Pat. No. 5,744,320, Australian Pat. No. 721172, Canadian Pat. No. 2,221,522 and other patents.

[e]U.S. Pat. Nos. 5,283,179, 5,641,641, 5,650,289 and 5,814,471, Australian Pat. No. 649289 and other patents.

[f]Certain applications of this product may require licenses from others.

[g]This product does not convey a license to use recombinant *Renilla* luciferase under U.S. Pat. Nos. 5,292,658, 5,418,155 and related patents. Promega sells licensed *Renilla* luciferase vectors, which may be used in conjunction with this product.

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