CELLTITER-GLO® ASSAY: APPLICATION FOR ASSESSING DIRECT CYTOTOXICITY AND FOR DETERMINING CELL PROLIFERATION

JÓZEFA WESIERSKA-GADEK, PH.D., AND JACEK WOJCIECHOWSKI, PH.D., CELL CYCLE REGULATION UNIT, INSTITUTE OF CANCER RESEARCH, UNIVERSITY OF VIENNA, VIENNA, AUSTRIA

The authors assess the performance of the CellTiter-Glo® Luminescent Cell Viability Assay in several applications. They show that the assay is linear over a broad range of cell densities, and they compare the CellTiter-Glo® Assay to a conventional colorimetric method for determining cell viability. Additionally, the authors use the CellTiter-Glo® Assay to assess direct cytotoxicity in HeLa Cells.

Introduction

The capacity of established cell lines to proliferate in cell culture depends primarily on the intrinsic status of the cells. Primary, nontransformed cells have lower proliferating potential than immortalized or transformed cells. Moreover, the kinetics of cell proliferation is characteristic for distinct cell lines. Some cell types grow relatively slowly after plating because they need cell-to-cell contact to stimulate exponential proliferation, whereas other cell types stop dividing after reaching higher cell density. In this case, contact inhibition may play an essential role in halting proliferation. Considering these aspects, scientists require methods for determining cell densities across a broad range of cell numbers. Furthermore, to understand the mechanisms of action of some drugs or agents, discriminating between direct cytotoxicity and inhibition of cell proliferation is necessary. To assess direct cytotoxic effects, cell viability should be determined at the end of continuous treatment with test compounds.

On the other hand, the inhibition of cell proliferation by distinct drugs becomes more evident if drugs are removed after treatment (i.e., cells are treated for 12 or 24 hours and then cultivated in drug-free medium for an additional 24 or 48 hours). The untreated controls will proliferate, but proliferation of treated cells will be inhibited, depending on the capacity of the treatment compound to block the cell cycle or to induce cell death. This protocol allows the long-term effects of the tested drugs to be visualized more easily. Recently, the CellTiter-Glo® Luminescent Cell Viability Assay[a] was developed. This assay method is based on direct determination of the intracellular ATP level and works across a broad range of cell densities, allowing researchers to assess direct cytotoxicity and to determine cell proliferation.

To prove the suitability of this new method for determining cell proliferation and to explore putative new applications, we address several important questions. First, we examine whether the CellTiter-Glo® Assay is linear within a broad range of cell densities and compare its sensitivity with a conventional colorimetric method. Second, we ask whether the intensity of luminescence generated by distinct cell lines depends on the cell size. Third, we address the question of whether this assay is designed to assess the direct cytotoxicity exerted by some drugs or genotoxic agents. Discriminating between direct cytotoxic effects and indirect effects that inhibit cell proliferation is necessary when considering the mechanism of action of test compounds.

Methods

Cell lines. We used human cervix carcinoma HeLa S3 cells, mouse fibroblasts (NIH3T3) and mouse embryo fibroblasts obtained from wildtype (A-19) and poly(ADP-ribose) polymerase-1 knockout (PARP-1-deficient, A-12 and A-11) mice (1).

Cell proliferation assays. Defined numbers of cells were plated in 96-well plates, and after a few hours the CellTiter-Glo® Assay or a conventional colorimetric proliferation assay (based on the determination of the cleavage of the tetrazolium salt WST-1 to formazan by cellular enzymes) was performed.

Cell treatment. In other experiments cells plated in 96-well plates or Petri dishes were grown to 60–70% confluency and then treated with 50µM N-methyl-N´-nitro-N-nitrosoguanidine (MNNG) for indicated periods of time.

Cell volume. Cells suspended in PBS were used for determination of cell volume. Three measurements were performed sequentially using the Cell Analyser System CASY1 TTC.

Dye exclusion test. The vital dye 7-amino-actinomycin D (7-AAD) was used. Viable cells with intact membranes exclude 7-AAD, but the membranes of dead and damaged cells are permeable to 7-AAD. Cells seeded in petri dishes were washed twice with cold PBS, and then 5µl of 7-AAD-staining solution (BD Biosciences) diluted in 1ml PBS was added to the cells. After 15 minutes, the cells were inspected by microscopy. Fluorescence was monitored in the far red range spectrum, and total cells were visualized by Hoffman modulation contrast (HMC).

Results and Discussion

CellTiter-Glo® Assay Results Are Linear Over a Wide Range of Cell Densities

We first measured the intensity of the luminescent signal generated by the CellTiter-Glo® Assay from precisely defined cell numbers of a few distinct cell lines. As shown in Figure 1 the intensity of luminescence was directly proportional to the cell numbers. Even a low number of cells generated clear and reproducible luminescent signals. We measured 35,000cpm for one thousand normal mouse fibroblasts.

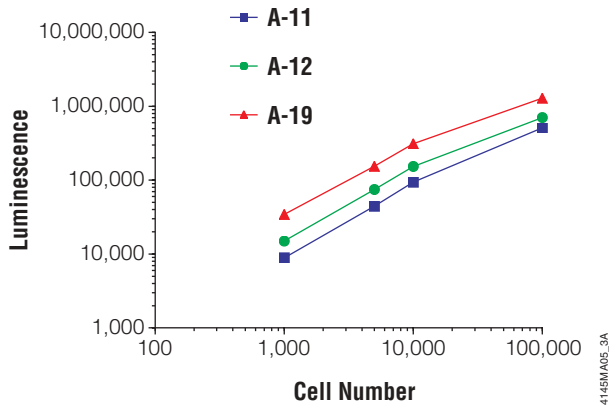

Figure 1. Linear relationship between the intensity of luminescence and cell number for different cell types. Mouse fibroblasts obtained from normal (A-19) and PARP-1-deficient mice (A-12 and A-11) were plated into 96-well plates at a defined cell number in a final volume of 100µl. CellTiter-Glo® Reagent (100µl) was added, and after rotation and incubation, the luminescence was measured according to the manufacturer's recommendations (*CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin* #TB288). Cells plated in 8 wells were measured for each determination. (Replicates varied by less than 6 percent.)

Next we compared a conventional colorimetric assay to the CellTiter-Glo® Assay. The conventional assay is based on reduction of the tetrazolium salt WST-1 to formazan by cellular enzymes. We used 1×10^4 cells as a standard value (100%) and compared the intensity of the absorbance and luminescence measured for the same cell numbers. As shown in Figure 2, the results of the colorimetric assay differed substantially from those of the CellTiter-Glo® Assay. The CellTiter-Glo® Assay revealed a linear relationship between cell numbers and the intensity of luminescence signal. However, results indicate that the conventional method is of limited use with low cell numbers. The lack of a good correlation between cell number and the absorbance appears to result from a high background signal in medium without cells. These results show that even a low number of cells can be measured by the CellTiter-Glo® Assay and that the deviations are low.

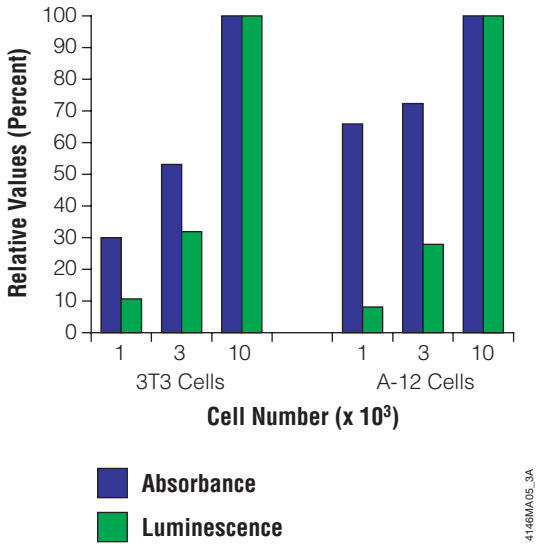

Figure 2. Comparison of the CellTiter-Glo® Assay with a conventional cell proliferation assay. The conventional assay measured the cleavage of the tetrazolium salt WST-1 to formazan by cellular enzymes. Mouse fibroblasts NIH3T3 and PARP-1-deficient A-12 cells were plated into 96-well plates at a defined cell number in a final volume of 100µl. CellTiter-Glo® Reagent (100µl) or 10µl of WST-1 reagent was added, and after mixing and incubation, the luminescence or absorbance was measured according to the manufacturer's recommendations. Cells plated in 4 wells were measured for each determination. "No-cell" background values of 900–1,100cpm were not subtracted from experimental values before plotting the relative percent values.

These results show that even a low number of cells can be measured by the CellTiter-Glo® Assay.

CellTiter-Glo® Assay Results and Cell Size

Different cell types differ in size. One might assume that large cells possessing more mitochondria would generate more ATP than smaller cells. To examine the relationship between cell size and the intensity of generated luminescence, we performed serial measurements with the same number of cells differing in size. To eliminate the cell-specific differences in the amounts of endogenous ATP, we used mouse fibroblasts from the same mouse strain. We compared fibroblasts obtained from normal mice and from mice in which the poly(ADP-ribose) polymerase-1 gene was disrupted. We reported a few years ago that the size of mutant fibroblasts was significantly reduced in the PARP knockout cells compared to the normal cells (2). Phase contrast microscopy revealed that the mutant cells (A-11, A-12) are smaller than the wildtype cells (A-19; Figure 3).

At comparable cell density, a larger number of mutant cells was scored per square unit. To exclude the possibility that the observed phenotypic difference was a consequence of a stronger bottom adherence of the wildtype MEFs or was due to their flatter shape, we determined the volume of suspended cells by CASY (Cell Analyser System CASY1 TTC). Measuring the cell volume and evaluating cell diameter revealed that the cell diameter of the mutant cells was reduced by 20–30% (Figure 4). Moreover, subtle size differences were also observed between two cell lines established from PARP-1 Knockout mice.

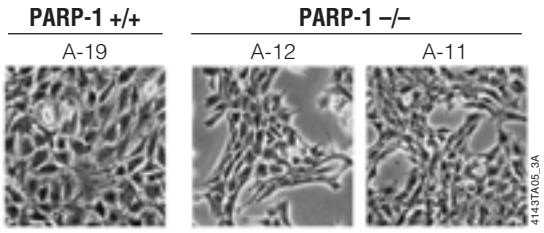

Figure 3. Differential phenotype of PARP-1-deficient cells. Phase contrast microscopy of living cells. Mouse fibroblasts obtained from normal (A-19) and PARP-1-deficient (A-12, A-11) mice were plated in petri dishes, and 24 hours after plating the cells were inspected under phase contrast microscopy and photographed.

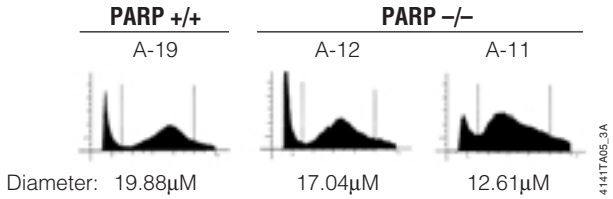

Figure 4. Reduced size of PARP-1 knockout cells. Normal (A-19) and PARP-1 KO (A-12, A-11) cells suspended in PBS were used for measurement of cell volume by Cell Coulter + Analyser CASY1 Model TTC. Cell suspension was introduced in a capillary of 30µM. Three sequential measurements were performed; about 5×10^3 cells were determined. The diameter evaluated for the majority of cells for each cell line is shown.

Interestingly, the determination of the intracellular ATP level revealed differences between cell lines that coincided with the cell size (Figure 5). The highest intensity of luminescence was generated by normal mouse cells, whereas mutant cells produced a signal of reduced intensity. A slight difference in luminescence also occurred between two mutant cell lines, A-12 and A-11. Although the differences in the intensity of luminescence generated largely reflected the cell size, the relationship between cell size and the amount of ATP was not directly proportional, indicating that the endogeneous ATP level is characteristic of distinct cell lines.

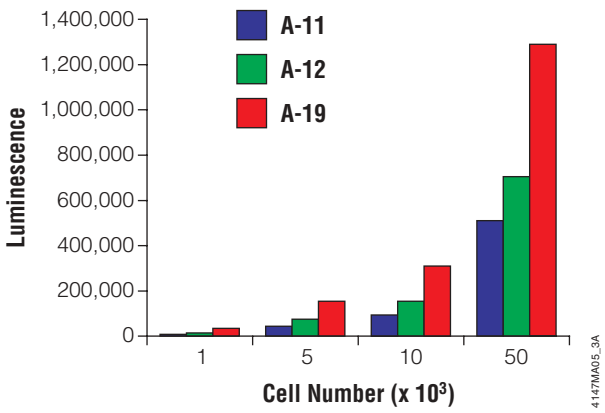

Figure 5. Relative ability of different cell lines to generate ATP-dependent luminescence. Mouse fibroblasts obtained from normal (A-19) and PARP-1-deficient (A-12, A-11) mice were plated into a 96-well plate at a defined cell number in a final volume of 100µl. One hundred microliters of CellTiter-Glo® Reagent was added, and after rotation and incubation, the luminescence was measured according to the manufacturer's recommendations. Cells plated in 8 wells were measured for each determination. "No-cell" background values of 900–1,100cpm were not subtracted from experimental values before plotting the relative percent values. (Replicates varied by less than 6 percent.)

Measuring Direct Cytotoxicity Using the CellTiter-Glo® Assay

We examined whether the CellTiter-Glo® Assay measures direct cytotoxicity and whether the dramatic activation of PARP-1 by alkylating agents, which leads to depletion of the cellular NAD pool, will generate a low luminescent signal. For this purpose we measured the viability of human cervix carcinoma HeLa cells treated with a high dose of the alkylating agent MNNG for 3 hours compared to untreated controls (Figure 6A). Cell viability as measured by the CellTiter-Glo® Assay dramatically decreased during the treatment and was diminished to 3%.

MNNG, especially at high doses, generates massive DNA strand breaks that activate the nuclear enzyme PARP-1, leading to the depletion of the cellular NAD pool. To ensure that the essential reduction of the luminescent signal reflects the loss of cell viability and is not a consequence of the extensive activation of PARP-1 by damaged DNA, we inspected the cells treated in a parallel assay in petri dishes by microscopy and determined the integrity of plasma membrane by a dye exclusion test. As depicted in Figure 6B, the exposure of HeLa cells to 50µM MNNG for 3 hours strongly affected the cell viability. A substantial number of cells lost their adherence and became floating. The residual adherent cells showed reduced density, and the majority of adherent cells accumulated 7-AAD, evidencing the loss of plasma membrane integrity. Moreover, we determined the viability of floating cells. Lack of luminescent signal in the

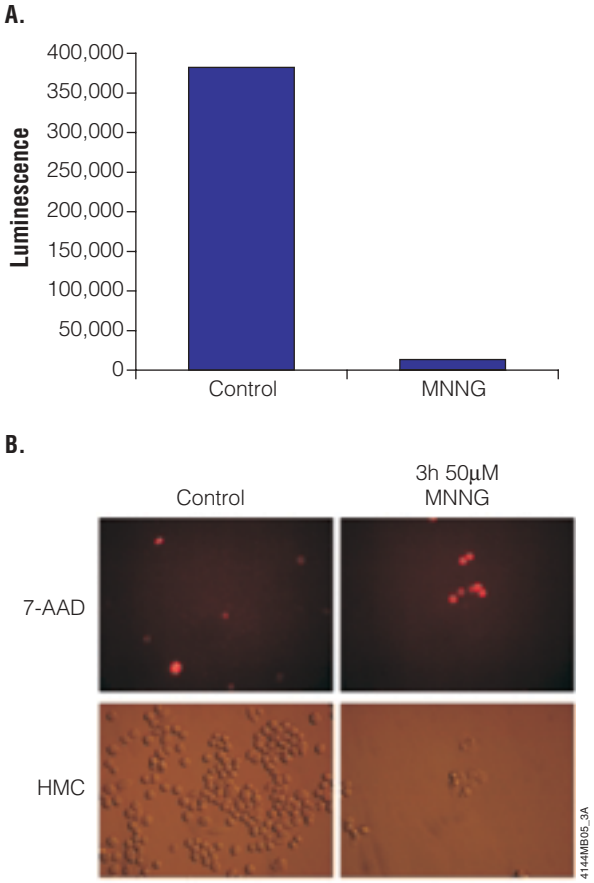

Figure 6. Measurement of direct cytotoxicity using the CellTiter-Glo® Assay. Panel A. Measurement of cell viability using the CellTiter-Glo® Assay of adherent HeLa S3 cells. HeLa cells plated into 96-well plates at a cell density of 5×10^3 were cultivated for 24 hours and then treated with 50µM MNNG for 3 hours. After treatment, control and treated cells were washed with PBS, and CellTiter-Glo® Reagent was added. After rotation and incubation, the luminescence was measured according to the manufacturer's recommendations. **Panel B.** Control HeLa cells and cells exposed for 3 hours to 50µM MNNG were gently washed with PBS and stained with 7-AAD (7-amino-actinomycin D). Cells were inspected under a light microscope using Hoffman modulation contrast (HMC) to visualize all cells present in the field, and damaged cells were detected by accumulation of vital dye 7-AAD.

fraction of floating cells closely correlated with dye accumulation (not shown). These results clearly show that the exposure of HeLa cells to MNNG disrupted the integrity of plasma membrane and impaired their viability. These changes were reflected by CellTiter-Glo® Assay. Thus, our results show that the CellTiter-Glo® Assay is also suitable for determination of direct cytotoxicity.

Conclusion

These results are important for several reasons. First, they show a close correlation between cell viability and the intensity of the luminescent signal determined by the CellTiter-Glo® Assay. Second, our results indicate that the CellTiter-Glo® Assay is designed to assess cell viability even for a low cell number. Additionally, some treatments of cells induce apoptosis. Induction of apoptosis in cell lines growing as a monolayer very frequently leads to cell shrinkage and loss of cell adhesion. In such a case exact determination of the number of cells that are floating and their viability is necessary. The CellTiter-Glo® Assay allows such measurements. Furthermore, for evaluating the inhibition of cell proliferation, an assay that is accurate over a broad range of cell numbers is indispensable. Our results substantiate that the CellTiter-Glo® Assay, which is based on the direct determination of intracellular ATP level, meets the criteria described above. ■

References

1. Wang, Z.-Q. *et al.* (1995) *Genes Dev.* **9**, 509–20.
2. Wesierska-Gadek, J. *et al.* (2000) *J. Cell. Biochem.* **78**, 681–96.

Protocol

CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin #TB288
(www.promega.com/tbs/tb288/tb288.html)

Ordering Information

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay[a]	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573

[a] Patent Pending.