

APPLICATION NOTE

Luminescent cell viability and cytotoxicity assays on the SpectraMax i3x Multi-Mode Microplate Reader

Introduction

Molecular Devices SpectraMax® i3x Multi-Mode Microplate Reader, in combination with luminescent assays for cell viability and cytotoxicity, provides a sensitive and rapid way to measure the number of viable cells in culture and quantify the cytotoxic effects of experimental treatment. The CellTiter-Glo assay from Promega uses luciferase enzyme, which requires ATP in order to generate light. The luminescent signal produced in the assay depends on the amount of ATP in the culture, which in turn depends on the number of viable cells present. The Bioluminescence Cytotoxicity Assay Kit from BioVision is based on the measurement of adenylate kinase (AK), a ubiquitous protein present in all cells and released into culture medium upon loss of plasma membrane integrity. AK converts ADP to ATP, which is then detected via a luminescent reaction.

Materials

- CellTiter-Glo Luminescent Cell Viability Assay (Promega P/N G7570)
- Bioluminescence Cytotoxicity Assay Kit (BioVision P/N K312-500)
- HeLa cells (ATCC P/N CCL-2)
- Black/clear 96-well tissue culture plates (Corning P/N 3904)
- Solid white 96-well tissue culture plates (Corning P/N 3917)
- Solid white 384-well tissue culture plates (Greiner P/N 781080)
- SpectraMax i3x Multi-Mode Microplate Reader

Methods

Preparation of reagents

CellTiter-Glo Buffer and substrate were thawed and equilibrated to room temperature prior to use. The contents of the bottle of CellTiter-Glo Buffer were transferred into the amber bottle containing CellTiter-Glo Substrate, and the contents were mixed by gentle inversion as directed in the kit's technical bulletin.

For the Bioluminescent Cytotoxicity Assay Kit, a vial of AK Detection Reagent was reconstituted with 1.1 mL of AK Assay Buffer, mixed gently, and allowed to equilibrate at room temperature for 15 minutes. This AK Stock Reagent was then diluted 10-fold into Assay Buffer to make the AK Detection Reagent Working Solution.

Correlating cell numbers with luminescent signal

HeLa cells were cultured in MEM supplemented with 10% fetal bovine serum penicillin/streptomycin. Cells were trypsinized, suspended in culture medium, and counted. For the 96-well assay format, serial cell dilutions from 50,000 to 10 cells per well were plated at 100 μ L of cell suspension per well. For the 384-well format, serial cell dilutions from 12,500 to 6 cells per well were plated at 25 μ L per well. Control wells containing medium without cells were prepared to obtain background luminescence values.

To ensure accurate cell numbers for the cell standard curves, cells were assayed immediately. 100 μ L (96-well assay) or 25 μ L (384-well assay) of CellTiter-Glo reagent was added to each well. The plate was mixed gently for 2 minutes on a plate shaker and then allowed to incubate at room temperature for 10 minutes to stabilize luminescence.

Benefits

- **Highly sensitive luminescence detection down to 10 cells per well**
- **Microplate and read height optimization wizards for best signal detection**
- **Preconfigured software protocols for faster analysis of results**

ATP standard curves

A 1:10 dilution series of ATP was set up in solid white 96- and 384-well plates at concentrations from 10 μ M down to 1 nM, along with blank wells containing no cells. The CellTiter-Glo assay was used to measure ATP. Microplate optimization and read height optimization were performed for best results.

Cell viability and cytotoxicity assays

HeLa cells were seeded at 15,000 cells per well in 96-well solid white or clear-bottom tissue culture-treated microplates and allowed to attach and grow overnight. The next day staurosporine and anisomycin were added to the cells to induce apoptosis. Compounds were added as a 1:2 dilution series starting at a top concentration of 50 μ M and going down to 24 nM. After 24 hours of treatment with compounds, cells were assayed with the CellTiter-Glo and BioVision Cytotoxicity kits.

For the cell viability assay, 100 μ L of CellTiter-Glo reagent was added to each well of the solid white plate containing treated cells. The plate was read about 10 minutes after reagent addition to allow for cell lysis.

For the BioVision cytotoxicity kit, 100 μ L of culture medium was removed from each treated well and transferred to a solid white plate. 100 μ L of AK Reagent Working Solution was added to each well, and the plate was read within 30 minutes.

Instrument setup and sample analysis

SpectraMax i3x reader settings were configured as indicated in Table 1.

Calculations of average RLU and standard deviations, graphing of cell dilution series and ATP standard curves, and 4-parameter curve fitting for IC_{50} curve generation were performed using SoftMax[®] Pro Software. A preconfigured protocol for the CellTiter-Glo assay is included in the software's protocol library.

Results

The SpectraMax i3x reader was able to detect as few as 10 cells per well in both 96-well and 384-well assay formats. Cells can thus be measured at numbers below the detection limits of standard colorimetric and most fluorometric methods. The CellTiter-Glo assay was linear over the entire range of cell concentrations used,

Parameter	Selected setting
Optical configuration	Monochromator
Read Mode	LUM
Read Type	Endpoint
Wavelengths	All Wavelengths
Plate types (specific to each assay format)	96 Well Costar clear 384 Well Greiner clear*
PMT and Optics	Integration Time: 1000 ms Read Height: 5.86 to 8.09 mm (assay dependent)**
More Settings	Show Pre-Read Optimization Options

*The 384-well microplate plate definition was optimized using the Microplate Optimization Wizard in SoftMax Pro Software.
**Read height was optimized for each assay using the Read Height Optimization Wizard to ensure optimal signal detection.

Table 1. Instrument settings for luminescence readings.

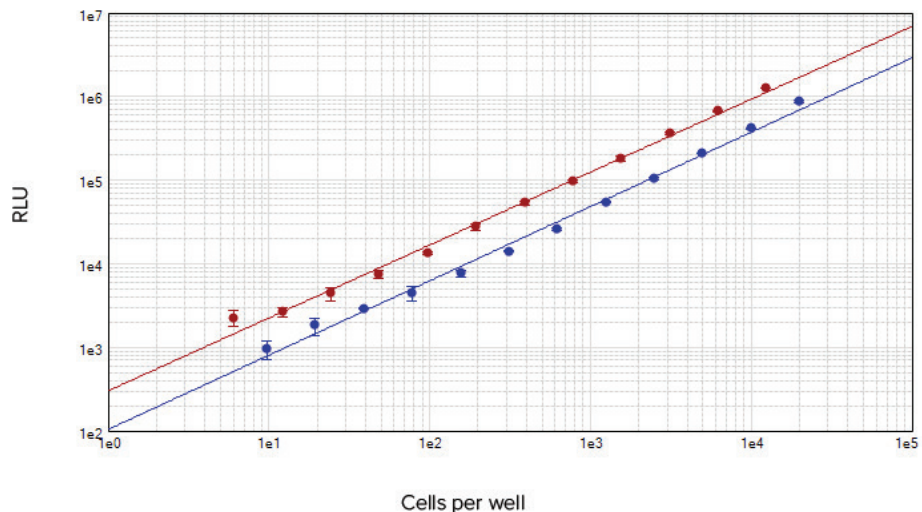


Figure 1. Cell standard curves for 96-well (blue circles) and 384-well (red circles) assay formats are shown. In each format, cell numbers at least as low as 10 cells per well can be detected. For both standard curves, $r^2 > 0.99$.

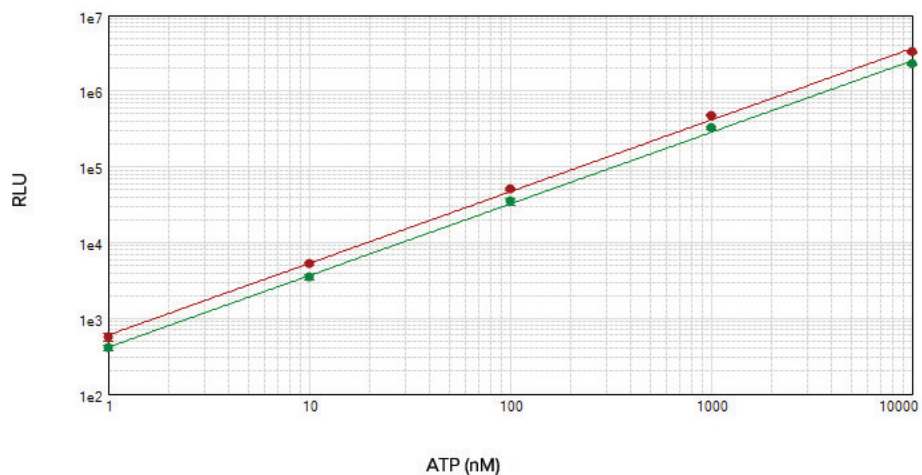


Figure 2. ATP standard curves for 96-well (red circles) and 384-well (green circles) assay formats are shown. For both standard curves, $r^2 = 0.999$.

spanning greater than three orders of magnitude with $r^2 > 0.99$ (Figure 1).

ATP standard curves can be used to verify assay performance and calculate cellular ATP content. Here, luminescent signal from ATP standards ranging from 1 nM to 10 μ M covered the entire range of luminescence output observed in the numbers of cells assayed here (Figure 2). Measurements were linear over at least four decades.

Cells treated with the compounds staurosporine and anisomycin were assayed for cell viability by measuring ATP levels (Figure 3), and for cytotoxicity by assaying for the enzyme adenylate kinase (Figure 4). Both assays yielded similar IC_{50} values for staurosporine (0.34 μ M and 0.15 μ M, respectively) and for anisomycin (3.30 μ M and 2.28 μ M).

Conclusion

The SpectraMax i3x reader offers excellent sensitivity in luminescence detection for ATP-based cell viability and adenylate kinase-based cytotoxicity assays, enabling detection of as few as 10 cells per well. Four-parameter curve fitting and calculation of IC_{50} values were all done within the SoftMax Pro Software. Microplate and read height optimization wizards ensure optimal signal detection and are automated by the software.

In addition to luminescence detection, the SpectraMax i3x reader gives users the capability of multiplexing the CellTiter-Glo and BioVision Cytotoxicity assays with other assays requiring additional detection modes. This multi-mode reader offers absorbance detection and fluorescence intensity detection with patent-pending Spectral Fusion™ Illumination to deliver wavelength flexibility while maximizing signal strength.

The ability to add user-exchangeable cartridges, including time-resolved fluorescence and western blot detection, and the optional SpectraMax® MiniMax™ 300 Imaging Cytometer module, expands the reader's detection capability and enables application options far exceeding those of other readers. All instrument configurations are operated using SoftMax Pro Software, which contains preconfigured protocols for CellTiter-Glo and over 120 other assays to facilitate data collection and analysis.

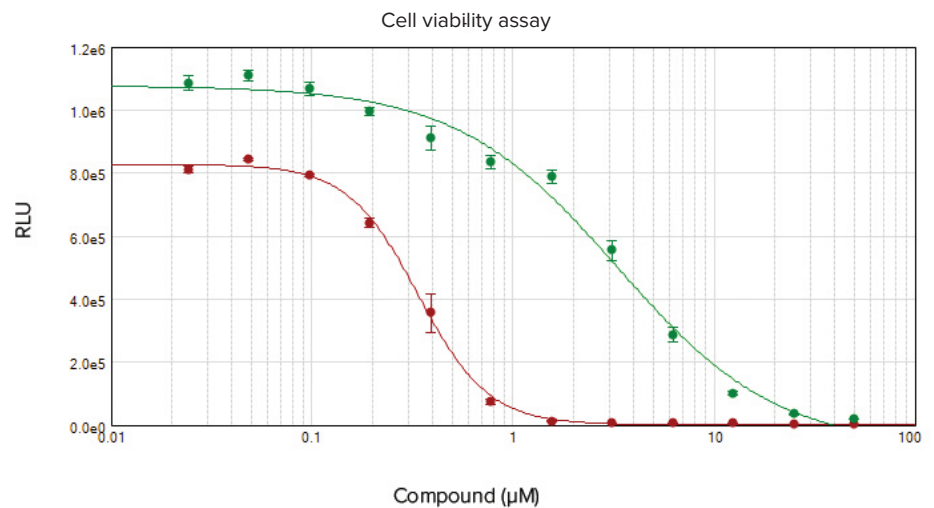


Figure 3. Concentration-response curves for HeLa cells treated with staurosporine (red circles) and anisomycin (green circles) for 24 hours. The IC_{50} value for staurosporine was 0.34 μ M, compared to 3.30 μ M for anisomycin.

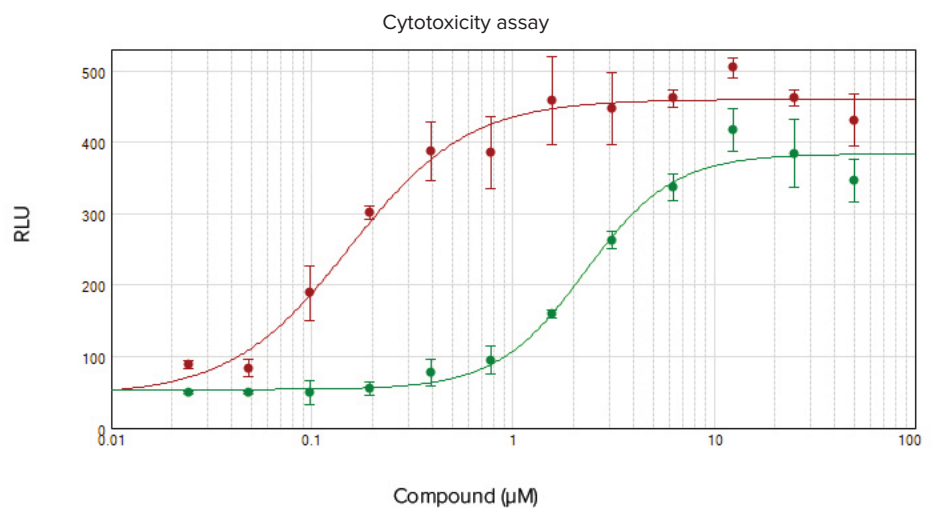


Figure 4. Concentration-response curves for HeLa cells treated with staurosporine (red circles) and anisomycin (green circles) for 24 hours. Cells were assayed for adenylate kinase activity using the BioVision Cytotoxicity Assay Kit. The IC_{50} value for staurosporine was 0.15 μ M, compared to 2.28 μ M for anisomycin.

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