APPLICATION NOTE

Measuring cell health on the SpectraMax iD3 reader with cell viability assays

Introduction

Measuring cell health from the multiple vantage points of viability and apoptotic pathways can provide insight into the impacts of a variety of experimental treatments including drug candidates, pathway activators and inhibitors, and reporter genes.

One of the most popular methods for assaying cell health is fluorescence detection with a microplate reader. In this application note, we report the use of the EarlyTox™ Cell Viability suite of assay kits on the SpectraMax® iD3 Multi-Mode Microplate Reader (Figure 1).

EarlyTox Live/Dead Assay Kit

The kit contains two markers for live or dead cells that are suitable for use with mammalian cells. Calcein AM is a widely used live-cell marker. The non-fluorescent calcein AM permeates the intact cell membrane and is converted into fluorescent calcein by intracellular esterases. Live cells are stained with intense green fluorescence in the cytosol. For cell proliferation assays, or other assays where live cell staining only is desired, calcein AM can be used as a standalone reagent as provided in the EarlyTox™ Live Cell Assay Kit (P/N R8342 for Explorer kit, P/N R8343 for Bulk kit).

Ethidium homodimer-III (EthD-III) is virtually non-fluorescent and impermeant to an intact plasma membrane. In the event of compromised cell membrane integrity that is associated with cell death, EthD-III enters cells and binds to nucleic acids, resulting in bright red fluorescence in dead cells. Cytotoxic events that affect cell membrane integrity can be accurately assessed using this method.

Fluorescent signals from calcein and EthD-III can be detected using the SpectraMax iD3 reader and rapidly analyzed using the EarlyTox Live Dead preconfigured protocol in SoftMax® Pro Software.

Benefits

- **Fully optimized assays for SpectraMax iD3 reader save time on assay development**
- **Simple, homogeneous workflow minimizes cell handling**
- **High-resolution touchscreen allows for quick set up of methods**
- **Preconfigured protocols in SoftMax Pro Software expedite data analysis**

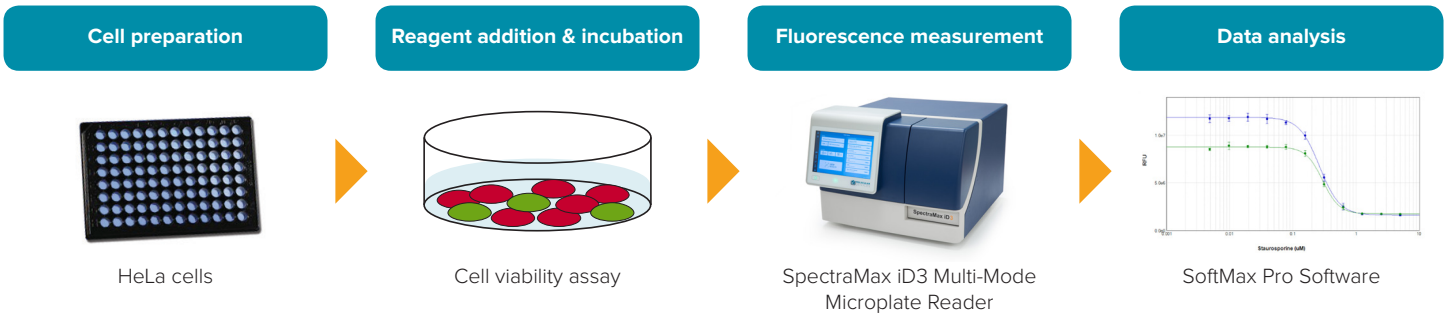

Figure 1. Representative experimental workflow for a cell viability assay.

EarlyTox Glutathione Assay Kit

This kit uses monochlorobimane (MCB), a cell permeant dye with a high affinity for GSH, to detect cellular GSH levels. Reaction of the dye with GSH is catalyzed by endogenous glutathione-S-transferase (GST) enzymes and results in the generation of blue fluorescence with excitation at 394 nm and emission at 490 nm. The fluorescence intensity corresponds to the amount of GSH present in cells, which increases with apoptosis. Unlike representative competitor assays, the EarlyTox Glutathione assay can be used on live, intact cells in a microplate format without the need for cell harvest and centrifugation, lysis, or other time-consuming manipulations that can lead to variability in results. Instrument settings and analysis in SoftMax Pro Software are simplified with the preconfigured EarlyTox Glutathione protocol.

EarlyTox Caspase-3/7 R110 Assay Kit

The EarlyTox Caspase-3/7 R110 Assay Kit provides a single-step, homogenous assay that is specifically designed for microplate readers. The fluorogenic substrate $(Ac\text{-}DEVD)_2$-R110 contains two DEVD consensus target sequence and is completely hydrolyzed in cell lysate by the enzymes in two successive steps. Hydrolysis of both DEVD peptides releases the green fluorescent dye rhodamine 110 (R110), resulting in a substantial fluorescence increase, with excitation at 490 nm and emission at 520 nm. A simplified workflow reduces the number of cells typically required for such an assay and also the variability typically encountered with multiple steps. A preconfigured EarlyTox R110 acquisition and analysis protocol is included in the SoftMax Pro protocol library.

Materials

- EarlyTox™ Live/Dead Assay Kit
  - Explorer Kit (2-plate size, Molecular Devices P/N R8340)
  - Bulk Kit (10-plate size, Molecular Devices P/N R8341)
- EarlyTox™ Glutathione Assay Kit
  - Explorer Kit (2-plate size, Molecular Devices P/N R8344)
  - Bulk Kit (10-plate size, Molecular Devices P/N R8345)
- EarlyTox™ Caspase-3/7 R110 Assay Kit
  - Explorer Kit (2-plate size, Molecular Devices P/N R8346)
  - Bulk Kit (10-plate size, Molecular Devices P/N R8347)
- HeLa cells (ATCC P/N CCL-2)
- Staurosporine (Sigma P/N S5921)
- 96-well black, clear-bottom microplates (Corning P/N 3904)
- SpectraMax iD3 Multi-Mode Microplate Reader

Methods

Cell treatment

HeLa cells were plated at 20,000 cells per well in 100 µL per well in a black, clear-bottom microplate. They were allowed to attach and grow overnight in a 37°C, 5% CO_2 incubator. The cells were then treated to induce apoptosis. For the Live/Dead assay, they were treated for 24 hours with 1:2 dilutions of staurosporine from 10 µM down to 40 nM. For the Glutathione and Caspase-3/7 R110 assays, they were treated for four hours with 1:2 dilutions of staurosporine from 5 µM down to 5 nM. Four replicates were run at each concentration.

EarlyTox Live/Dead Assay Kit

A 2X working solution of calcein AM/EthD-III was prepared by adding calcein AM and EthD-III stock solutions to PBS for a concentration of 6 µM for each dye. 100 µL of the 2X working solution was added to each assay well, resulting in a final volume of 200 µL and a final concentration of 3 µM for each dye. The plate was incubated at room temperature for one hour. It was then read from the top and bottom on the SpectraMax iD3 reader using a preconfigured protocol in SoftMax Pro Software with the settings indicated in Table 1. Note: removal of medium, followed by addition of a 1X solution of calcein AM and EthD-III, is optional and can help reduce background fluorescence if necessary.

The preconfigured EarlyTox Live Dead assay protocol in SoftMax Pro Software automatically calculated green/red ratios, which were then plotted against compound concentration. The protocol enables calculation of percentage of live and dead cells in the experimental cell samples where such analysis is required. For these calculations it is necessary to set up additional controls in the assay plate.

EarlyTox Glutathione Assay Kit

A 20 µM MCB working solution was prepared by diluting 20 µL of 10 mM MCB stock solution in 10 mL of PBS. Medium was removed from the cells in the assay plate and replaced with 100 µL of MCB working solution. Cells were incubated at 37°C. At one hour and two hours after reagent addition, fluorescence intensity was measured on the SpectraMax iD3 reader using the settings indicated in Table 1.

Parameter	EarlyTox Live/Dead	EarlyTox Glutathione	EarlyTox Caspase-3/7 R110
Read mode	Fluorescence	Fluorescence	Fluorescence
Read type	Endpoint	Endpoint	Endpoint
Wavelengths	Lm1: Ex = 495 nm, Em = 530 nm Lm2: Ex = 530 nm, Em = 645 nm	Ex = 394 nm, Em = 490 nm	Ex = 470 nm, Em = 520 nm
PMT and Optics	PMT gain: Automatic Integration time: 500 ms Read from bottom	PMT gain: Automatic Integration time: 500 ms Read from top or bottom	PMT gain: Automatic Integration time: 500 ms Read from top or bottom

Table 1. Settings for SpectraMax iD3 Multi-Mode Microplate Reader. Similar settings will work for other SpectraMax readers with fluorescence detection.

EarlyTox Caspase-3/7 R110 Assay Kit

Substrate assay buffer was prepared by adding enzyme substrate $(AC\text{-}DEVD)_2$-R110 (2 mM) to cell lysis/assay buffer at a ratio of 50 µL to 1 mL buffer. 100 µL of substrate assay buffer was added to each well, resulting in a final volume of 200 µL per well and a final concentration of 50 µM substrate. The samples were then incubated at room temperature. Fluorescence was measured on the SpectraMax iD3 reader at one hour and two hours after reagent addition using the settings indicated in Table 1.

Results

EarlyTox Live/Dead Assay Kit

HeLa cells treated with staurosporine showed a clear concentration response, which was easily quantified as the ratio of live (green fluorescence) to dead (red fluorescence) signal (Figure 2). Overall, RFU values were higher for cells incubated for two hours than for cells incubated for one hour (data not shown). The EC_{50} value calculated from a 4-parameter curve fit of the data was 300 nM.

EarlyTox Glutathione Assay Kit

HeLa cells treated with staurosporine for four hours exhibited an apoptosis-associated decrease in intracellular glutathione that was measured using the EarlyTox Glutathione assay. A decrease in fluorescence with increasing staurosporine concentration was detected with the SpectraMax iD3 reader. Results were graphed in SoftMax Pro Software using a 4-parameter curve fit. Fluorescence intensity values increased over time, but similar EC_{50} values of 263 nM and 295 nM were obtained for assay incubation times ranging from one to two hours (Figure 3).

EarlyTox Caspase-3/7 R110 Assay Kit

HeLa cells treated with staurosporine for four hours exhibited an apoptotic response that was measured using the EarlyTox Caspase-3/7 R110 assay. Fluorescence corresponding to apoptotic cells was detected with the SpectraMax iD3 reader. Results were graphed in SoftMax Pro Software using a 4-parameter curve fit. Cells incubated in substrate for one or two hours had similar EC_{50} values of 193 nM and 200 nM (Figure 4).

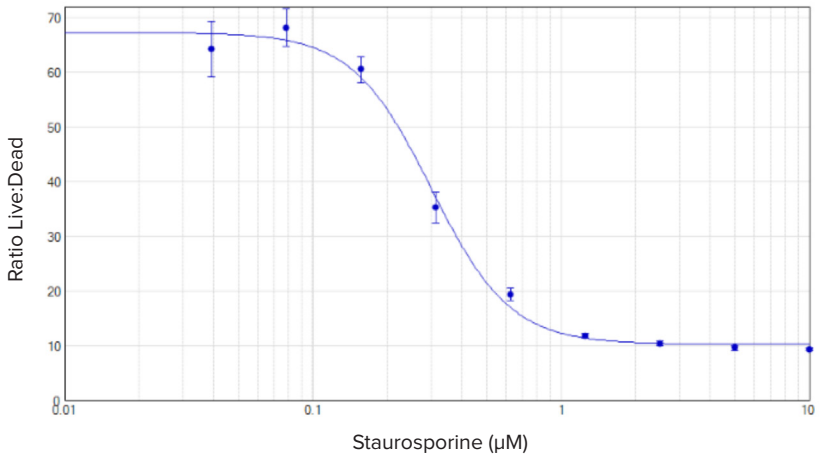

Figure 2. EarlyTox Live/Dead assay: concentration response curves for HeLa cells treated with staurosporine for 24 hours. Cells were incubated in calcein AM and EthD-III for one hour. Concentration curve was plotted using the ratio of green (530 nm emission) over red (645 nm emission) RFUs at the Y-axis. A 4-parameter curve fit was applied in SoftMax Pro Software. EC_{50} value was 300 nM.

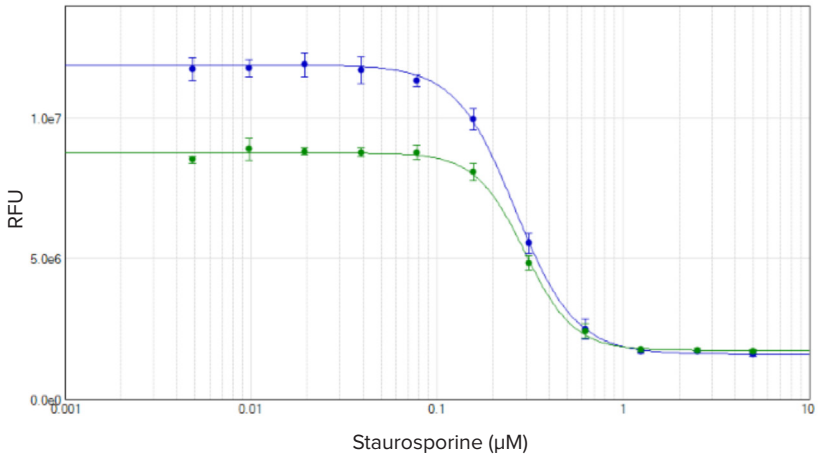

Figure 3. EarlyTox Glutathione assay: concentration response curves for HeLa cells treated with staurosporine for four hours. Cells were incubated in reagent for one (green plot) or two (blue plot) hours. Concentration curves were plotted using a 4-parameter curve fit in SoftMax Pro Software. EC_{50} values were 263 nM and 295 nM, respectively.

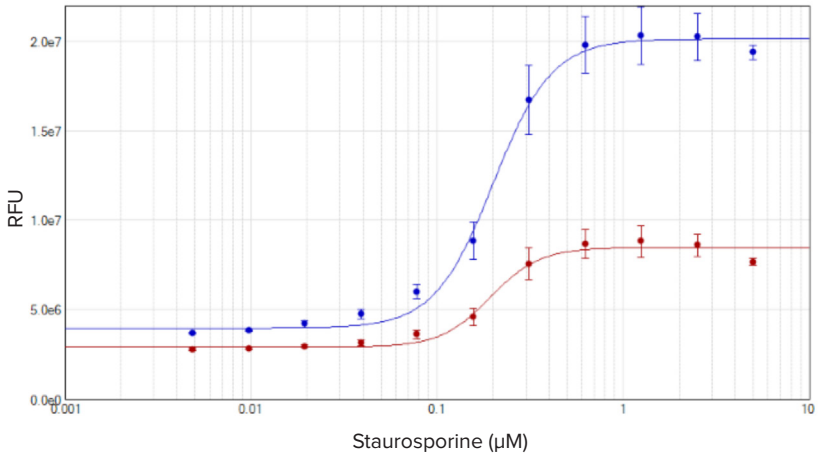

Figure 4. EarlyTox Caspase-3/7 R110 assay: concentration response curves for HeLa cells treated with staurosporine for four hours. Cells were incubated in reagent for one (red plot) or two (blue plot) hours. Concentration curves were plotted using a 4-parameter curve fit in SoftMax Pro Software. EC_{50} values were 193 nM and 200 nM, respectively.

Conclusion

Used together with the SpectraMax iD3 reader, the EarlyTox Cell Viability Assay Kits enable direct measurement of live and dead or apoptotic cells with a simple workflow and the increased throughput offered by a microplate format. Consistent results are obtained over time offering workflow flexibility. Preconfigured SoftMax Pro Software protocols provide optimized instrument settings and speed the time to results with automatic data analysis.

SpectraMax iD3 Multi-Mode Microplate Reader

The SpectraMax iD3 reader features a large, high-resolution touchscreen interface with an embedded software package allowing users to set up custom protocols, use pre-loaded protocols, and run experiments without the need for a dedicated computer workstation. Built-in near-field communication (NFC) functionality enables access to custom protocols and results with a single tap, saving precious time.

Contact Us

Phone: +1-800-635-5577
Web: www.moleculardevices.com
Email: info@moldev.com
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