

## APPLICATION NOTE

# Increase accuracy and efficiency of cytotoxicity assessment using the EarlyTox Live/Dead Assay Kit and high-content imaging

## Introduction

Cell viability assays are utilized in a wide variety of research areas ranging from examining the mechanisms of cell death and cell proliferation to evaluating the effects of cytotoxic compounds and novel therapies, including drug candidates, pathway activators and inhibitors, and genetic reporters. High-quality image acquisition combined with complete multi-parameter, high-throughput screening provides an optimal method to assess cell viability. In this application note, we report the use of the EarlyTox™ Live/Dead Assay Kit in conjunction with the ImageXpress® Micro High-Content Imaging System and the MetaXpress® High-Content Image Acquisition and Analysis Software.

The EarlyTox Live/Dead assay kit contains two markers for live cells or dead cells that are suitable for use with mammalian cells. Their unique mechanisms of action are dependent upon cell membrane permeability. Viable cells are stained with intense green fluorescence in the cytosol, with Calcein AM, a widely used live cell marker. The non-fluorescent Calcein AM permeates the intact cell membrane where the acetoxymethyl (AM) group is cleaved by intracellular esterases, thus yielding the fluorescent calcein molecule. Calcein AM can be used as a standalone reagent for cell proliferation assays or other assays where only live cell staining is desired, as provided in the EarlyTox™ Live Cell Assay Kit (Molecular Devices 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 and that eventually lead to cell death can be accurately assessed using this method.

The assay kit enables characterization of a full concentration-response profile of test compounds since the no-wash, homogeneous assay protocol eliminates the potential to remove dead and dying cells. Fluorescent signals from calcein and EthD-III can be detected using the ImageXpress Micro system and rapidly analyzed using the standard Live/Dead application module in MetaXpress software.

## 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)
- HeLa cells (ATCC P/N CCL-2)
- HeLa media
  - Minimum Essential Medium complete media with glutamine and serum
- CHO-M1WT3 cells (ATCC P/N CRL-1985)
- CHO media
  - Ham's F-12 complete media with glutamine and serum
- Staurosporine (Sigma P/N S5921)
- Mitomycin C (Sigma P/N M4287)
- 96-well black, clear-bottom microplates (Greiner P/N 655090)

## Benefits

- Implement a no-wash homogeneous assay protocol
- Shorten exposure times for rapid image acquisition
- Increase assay accuracy and efficiency with high-content imaging
- Utilize preconfigured Live/Dead analysis module in MetaXpress software

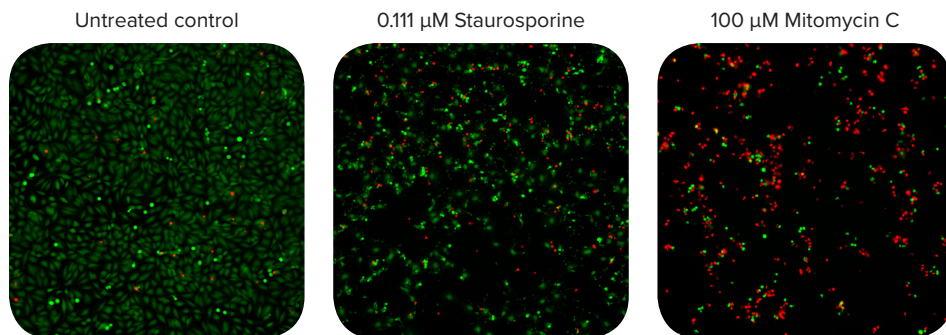
- 384-well black, clear-bottom microplates (Corning Falcon P/N 62406-490)
- ImageXpress Micro High-Content Imaging System with MetaXpress High-Content Image Acquisition and Analysis Software

## Methods

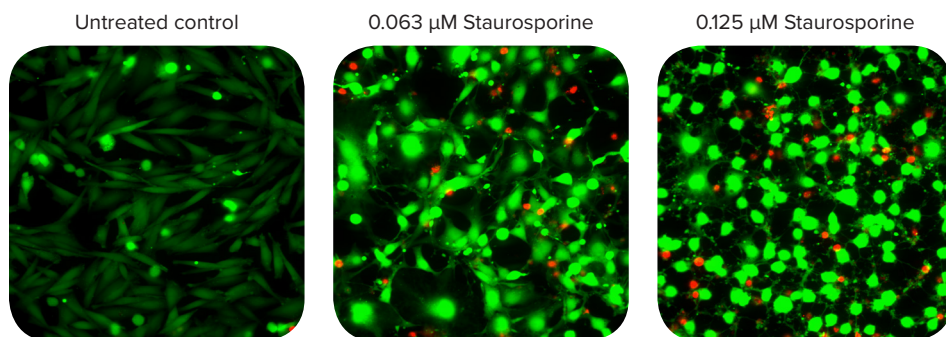
HeLa cells were plated in a 96-well microplate at 6,000 cells per well in a volume of 100  $\mu\text{L}$  per well. In a 384-well microplate, CHO-M1 cells were plated at 5,000 cells per well in a volume of 50  $\mu\text{L}$  per well. Both plates were placed in an incubator at 37°C, 5%  $\text{CO}_2$  overnight to allow for cellular adhesion and growth. Both cell types were then treated for 24 hours with staurosporine (general protein kinase inhibitor and a potential anti-cancer therapeutic).

HeLa cells were also treated with mitomycin C (potent DNA crosslinker and chemotherapeutic). The HeLa cells were treated in quadruplicate with compounds serially diluted 1:3, while the CHO cells were treated in triplicate with staurosporine serially diluted 1:2. 100  $\mu\text{L}$  of 2X compound dilutions were added to corresponding wells in the HeLa plate, except for control wells. The final concentrations of staurosporine were 1  $\mu\text{M}$  down to 0.000017  $\mu\text{M}$  (0.017 nM), and the final concentrations of mitomycin C were 100  $\mu\text{M}$  down to 1.694  $\mu\text{M}$ . 50  $\mu\text{L}$  of 2X staurosporine dilutions were added to the wells in the CHO plate for final concentrations of 2  $\mu\text{M}$  down to 0.00024  $\mu\text{M}$  (0.24 nM). Serially diluting the compounds enabled analysis of dose-dependent responses to the cytotoxic effects of these compounds.

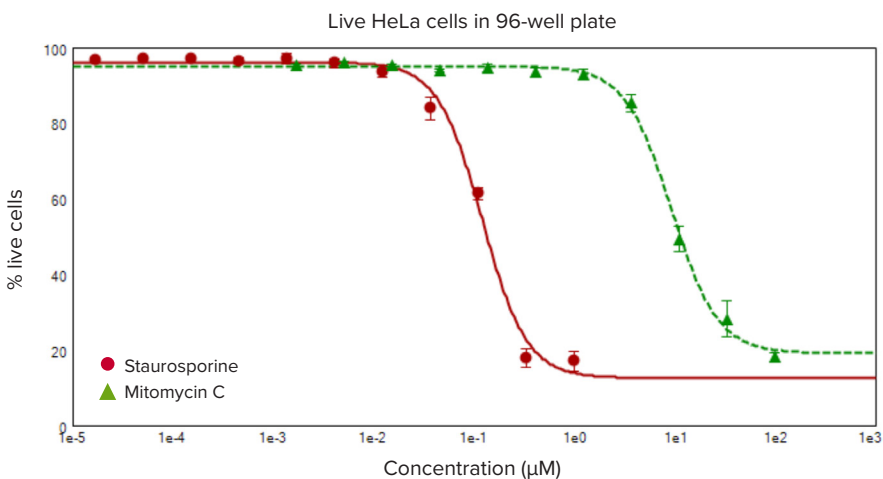
A 2X stain working solution was prepared by combining Calcein AM and EthD-III stock solutions in warm DPBS for a concentration of 2  $\mu\text{M}$  for each dye. Prior to the addition of the stain solution, an equal volume of media was removed from each well. This resulted in final concentrations of 1  $\mu\text{M}$  for each dye. The plates were incubated at 37°C, 5%  $\text{CO}_2$  for 45 minutes. In addition to the EarlyTox Live/Dead kit, a nuclear stain such as Hoechst 33342 can be added to the cells to aid in acquiring total cell count data. After the incubation for the Live/Dead stains, 10  $\mu\text{L}$  of a 55  $\mu\text{M}$  (11X) Hoechst working solution was spiked into each well for a final concentration of



**Figure 1. 10X widefield images of Calcein-AM stained (green) and Ethidium Homodimer-III stained (red) HeLa cells. (Left)** Untreated control, **(Middle)** 0.11  $\mu\text{M}$  staurosporine treated cells that exhibit a high incidence of dead cell nuclei; **(Right)** 100  $\mu\text{M}$  mitomycin C treated cells exhibiting a high incidence of dead cell nuclei.



**Figure 2. 40X widefield images of Calcein-AM stained (green) and Ethidium Homodimer-III stained (red) CHO cells. 100% of cells are identified as alive in the untreated control (Left) while Staurosporine treated wells exhibit a high incidence of dead cell nuclei 20% (Middle) and 27% (Right).**



**Figure 3a. Concentration response curves for percent of live HeLa cells treated with staurosporine (red) and mitomycin C (green) for 24 hours.** Live cells fluoresced green due to Calcein AM staining, and were counted with the Live/Dead analysis module in MetaXpress software. The cells were imaged at two sites per well by the ImageXpress Micro system, and the data from the two sites were averaged. The percent of live cells versus the concentration of compound was plotted with 4-parameter curve fit, which produced  $\text{EC}_{50}$  values of 0.121  $\mu\text{M}$  for staurosporine and 9.307  $\mu\text{M}$  for mitomycin C.

5  $\mu\text{M}$  per well. The cells were incubated at 37°C, 5%  $\text{CO}_2$  for 15 minutes longer.

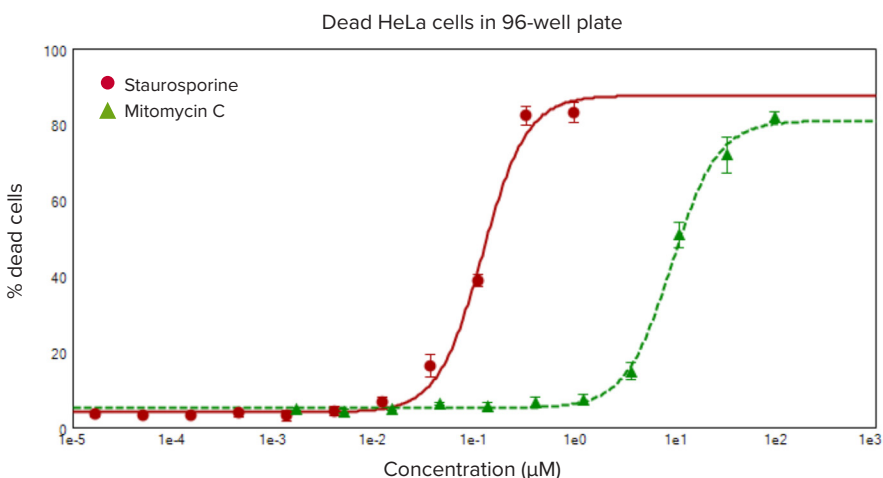
Immediately after the final incubation, the plates were imaged on the ImageXpress Micro system using a 10X Plan Apo objective and the FITC, Texas Red, and DAPI (since the nuclei were counterstained with Hoechst) filter sets. At this magnification, one field-of-view is capable of capturing 1,100-1,500 cells so that a single image yields statistically relevant results. The HeLa cell plate was imaged at two sites per well, which enabled analysis of >2000 cells/well. Images were taken using the widefield imaging mode (Figures 1 & 2).

### Bright staining enables fast read times

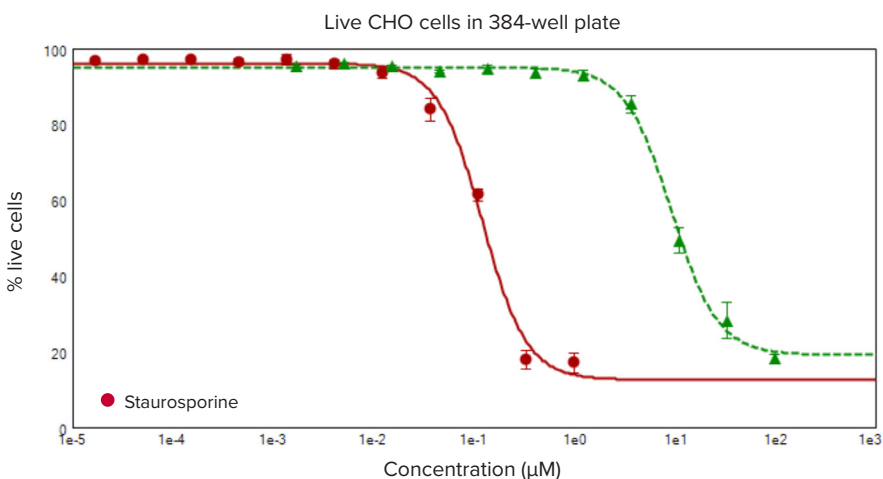
Capturing 10X images using the ImageXpress Micro system was highly efficient, as it took 2 ½ minutes to run an entire 96-well plate and 9 ¼ minutes to run a 384-well plate in 2 colors (FITC, Texas Red). The bright intensity of the fluorophores in the kit allowed the use of short exposure times, which enabled rapid image acquisition and prevented unwanted photobleaching.

### Preconfigured image analysis modules facilitate accurate data generation

The widefield images were analyzed using the Live/Dead application module in MetaXpress software. With intuitive user input, the module was configured to identify and differentiate live and dead cells based on the stain each cell type expressed. Multiple measurement parameters, such as the number of live and dead cells, percent of live and dead cells, total cell count, and intensity of each wavelength could be assessed. Furthermore, if nuclei are counterstained with Hoechst, a Multi-Wavelength Cell Scoring software module can be run along with (or instead of) the Live/Dead module. This provides additional analyses for the cell viability assay, such as scoring cells positively fluorescing at specific wavelengths (FITC or Texas Red) as well as accurately counting all nuclei.



**Figure 3b. Concentration response curves for percent of dead HeLa cells treated with staurosporine (red) and mitomycin C (green) for 24 hours.** Dead cells fluoresced red due to Ethidium Homodimer-III staining and were imaged at two sites per well by the ImageXpress Micro system. Cells were counted using a modified Live/Dead module in MetaXpress software, and the data from the two sites were averaged. The percent of dead cells versus the concentration of compound was plotted with 4-parameter curve fit, which produced  $\text{EC}_{50}$  values of 0.121  $\mu\text{M}$  for staurosporine and 9.307  $\mu\text{M}$  for mitomycin C.



**Figure 4a. Concentration response curve for percent of live CHO cells treated with staurosporine (red) for 24 hours.** Live cells fluoresced green due to Calcein AM staining and were imaged at a single site per well by the ImageXpress Micro system. The number of live cells was counted with the Live/Dead analysis module in MetaXpress software. A 4-parameter curve fit for the percent of live cells versus the concentration of compound was plotted and produced an  $\text{EC}_{50}$  value of 0.180  $\mu\text{M}$ .

## Toxicity $EC_{50}$ values can be calculated from dose-response curves

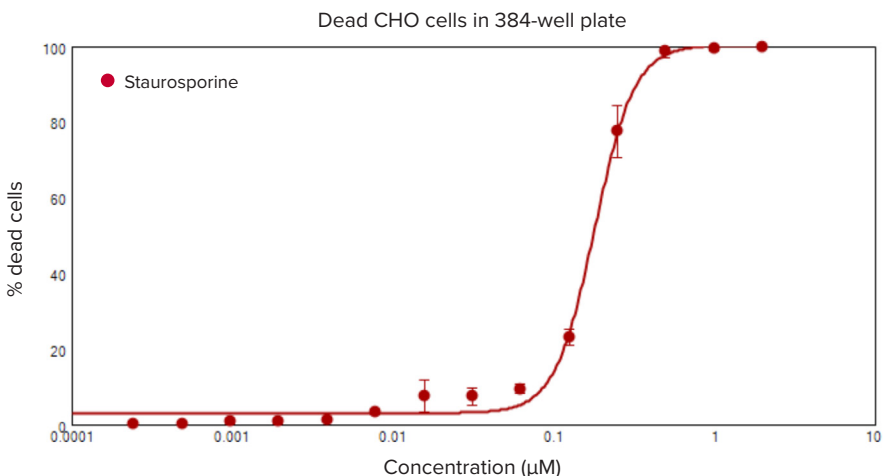
Live and dead cells were imaged and quantitative analysis was run based on cells staining positive for either calcein (green fluorescence) or with EthD-III (red fluorescence) (Figures 1 and 2). Treatments of HeLa cells with staurosporine and mitomycin C both showed clear concentration responses (Figures 3a and 3b). Graphs for percent of live cells and percent of dead cells vs. compound concentration produced dose response curves with  $EC_{50}$  values of 0.121  $\mu$ M for staurosporine and 9.307  $\mu$ M for mitomycin C. The same can be said for the CHO cells treated with staurosporine, as they too exhibited a distinct dose-dependent response (Figure 4a and 4b). The curves for the percent of live and dead CHO cells vs. staurosporine concentration generated  $EC_{50}$  values of 0.180  $\mu$ M. The comparable  $EC_{50}$  values for the staurosporine treatments in both experiments further demonstrated the accuracy of the EarlyTox reagents in the determination of live and dead cells.

## Conclusion

The EarlyTox Live/Dead Assay Kit, in conjunction with the ImageXpress Micro system with MetaXpress software, enabled an accurate measurement of live and dead cells with a highly efficient workflow. High-throughput screening technology offered a fast and robust image-based quantitation of the cell viability markers from the EarlyTox Live/Dead kit. MetaXpress software eliminated common assay artifacts which aided in producing cleaner and more accurate results. The consistent and statistically relevant quantitative analysis allowed for the testing of multiple compounds at differing concentrations and can be adapted to nearly any cell viability assay.

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**Figure 4b. Concentration response curve for percent of dead CHO cells treated with staurosporine (red) for 24 hours.** Dead cells fluoresced red due to Ethidium Homodimer-III staining and were imaged at a single site per well by the ImageXpress Micro system. The number of dead cells was counted with the Live/Dead analysis module in MetaXpress software. The percent of live cells versus the concentration of compound was plotted with a 4-parameter curve fit, which produced an  $EC_{50}$  value of 0.180  $\mu$ M.