

CellPlayer™ 96-Well Kinetic Caspase-3/7 Apoptosis Assay

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Introduction

Apoptosis, the biological process by which cells undergo programmed cell death, is required for normal tissue maintenance and development. However, aberrations in apoptotic signaling networks are implicated in numerous human diseases including neurodegeneration and cancer[1]. Apoptotic pathways are initiated by extrinsic factors that result in activation of pro-apoptotic receptors on the cell surface, or intrinsically by many different stimuli such as DNA damage, hypoxia, the absence of growth factors, defective cell cycle control, or other types of cellular stress that result in release of cytochrome C from the mitochondria.

Stimulation of either the extrinsic or intrinsic apoptotic pathways triggers a signaling cascade that results in the activation of a family of proteins that play a major role in carrying out the apoptotic process called caspases[2]. Caspases (cysteinyl aspartate proteinases) cleave substrates following an Asp (D) amino acid residue. Effector targets of caspases include caspase family members themselves, proteins involved in fragmentation of cellular DNA (Caspase Activated DNAses), nuclear lamins, as well as proteins that make up the cell cytoskeleton. Caspase proteins are traditionally separated into two groups, initiator caspases (caspase 2, 8, 9 and 10), and executioner or effector caspases (caspase 3, 6, and 7). As a primary executioner caspase in most systems, the activation of caspase-3 often results in the irreversible commitment of a cell to apoptosis. Therefore, the activation of caspase-3 is considered a reliable marker for cells undergoing apoptosis.

Numerous *in vitro* assays have been designed to measure the activation of caspase-3. The majority of these assays utilize reagent substrates that incorporate the DEVD (Asp-Glu-Val-Asp) motif which is recognized by both activated caspases 3 and 7[3]. This motif has been incorporated into luciferase, colorimetric, and fluorometric substrates that can be used in a variety of assay types, all of which result in only a single, user-defined time point measurement of caspase-3/7 activity. In addition, these techniques require multiple wash steps or cell lifting prior to data collection; potentially resulting in the loss of cells or critical data in experiments where cells undergo apoptosis at different rates according to treatment conditions.

Following the work of Daya et.al.[4], we introduce an optimized assay system incorporating the Essen CellPlayer™ Kinetic Caspase-3/7 Apoptosis reagent (kinetic apoptosis reagent) for use on the IncuCyte™ FLR imaging system. When added to the tissue culture growth medium, this inert, non-fluorescent substrate freely crosses the cell membrane where it is cleaved by activated caspase-3/7 resulting in the release of the DNA dye and green fluorescent labeling of DNA[5].

We provide evidence that this stable, homogeneous “mix and read” live cell reagent can be added directly to cells in culture without wash steps or cell lifting in order to kinetically, over multiple days, measure the induction of caspase-3/7 mediated apoptosis in a 96-well microplate format. Activation of caspase-3/7 is quantified using live-cell, time-lapse fluorescent images and the IncuCyte™ FLR fluorescent object counting algorithm. Additionally, phase contrast images can be used to validate associated morphological changes characteristic of apoptosis in the same cells over the same time course.

Approach and Methods

Cell culture and assay procedure

Prior to beginning the assay, cells were grown to confluence in 25 cm² tissue culture-treated flasks. MDA-MB-231 and MCF-7 cells were cultured in F12-K (Gibco) supplemented with Pen-Strep, 10% FBS, and 2 mM GlutaMAX (Gibco). HUVECs were cultured in complete Lonza EGM-2 BulletKit and were grown no further than passage 6. The day before starting the assay, cells were plated at 2500 cells/well (HUVEC) or 5000 cells/well (MDA and MCF-7) in a 96-well plate. Cells were allowed to adhere and grow overnight so that they were at ~25-35% confluence at the start of the assay. Prior to addition of the kinetic apoptosis reagent and/or treatment conditions, HUVECs were starved for two hours in 0.2% serum with no additional growth factors. SSP or Taxol were serially diluted with F12-K growth medium (100 µl per well) containing the kinetic apoptosis reagent at a final concentration of 5 µM. DMSO did not exceed 0.7% and did not affect proliferation or cell morphology relative to complete medium (data not shown). Cells were placed in an



IncuCyte™ FLR with a 10X objective in a standard cell culture incubator at 37 °C and 6% CO₂. Two images per well were collected every 2-3 hours in both phase-contrast and fluorescence. The assay was considered complete when a maximal response was achieved as determined by image analysis. For DNA object count at the assay end point, all DNA containing objects were labeled using the DNA-binding dye Vybrant DyeCycle Green (Life Technologies). Dye was diluted in PBS and added directly to medium-containing wells to a final concentration of 1 μM. Cells were incubated at 37 °C for 1-2 hours prior to imaging.

Data quantification and analysis

Throughout the assay, both phase and fluorescent images were collected, detecting both morphological hallmarks of apoptosis and caspase-3/7 activity, respectively. The integrated object counting algorithm was used to isolate the fluorescent nuclear signal from background, segment the signal into individual objects, and count objects on a per area basis for each time point. Because this reagent labels DNA and it is known that nuclear fragmentation is a hallmark of apoptosis, in many cases there is not a linear relationship of cell nuclei to counted objects. As a result, we have also successfully used the object confluence metric (the percentage of the image occupied by fluorescent objects) to kinetically quantify caspase-3/7 activity in this assay (see additional technical note). Both object count and object confluence metrics result in similar kinetic curves. Therefore, object count is presented throughout this application note. To correct for differential proliferation of cells, the total number of DNA containing objects was counted at the final time point using Vybrant Green. This number was used to calculate the “apoptotic index”, defined as the number of caspase-3/7 positive objects divided by the total number of DNA containing objects.

Results and Discussion

The kinetic activation of Caspase-3/7 can be quantitatively measured using the Essen CellPlayer™ Kinetic Caspase-3/7 Apoptosis reagent in the IncuCyte™ FLR.

The first experiment using the kinetic apoptosis reagent was designed to illustrate our ability to detect cells with activated caspase-3/7 using a well-known inducer of apoptosis, the general protein kinase inhibitor staurosporine (SSP). To accomplish this, we treated MDA-MB-231 cells, a human breast adenocarcinoma derived cell line, with SSP serially diluted in growth media containing 5 μM kinetic apoptosis reagent in a 96-well plate. Once treated, the cells were placed inside the IncuCyte™ FLR imaging platform with a 10X objective in a standard cell culture incubator and both phase

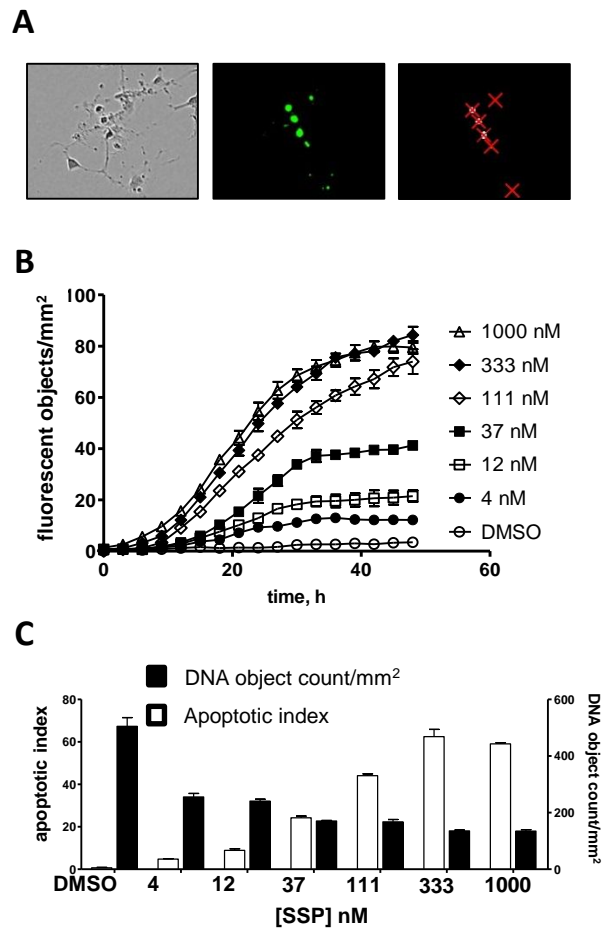


Figure 1: Staurosporine (SSP) induced caspase-3/7 activity in human breast adenocarcinoma cells. (A) Representative phase contrast and fluorescent images reveal classical apoptotic cell morphologies and indicate activation of caspase-3/7, respectively. The customizable object counting algorithm identifies fluorescent objects as indicated with red Xs. (B) Kinetic measures of the number of caspase-3/7 positive cells is recorded over time and plotted as fluorescent objects, n=3 wells per data point shown (C) At the 48 hour end point, the apoptotic index was calculated by dividing the number of Caspase-3/7 fluorescent objects by the total number of DNA containing objects following staining with Vybrant DyeCycle Green.

contrast and fluorescent images were collected every 3 hours.

Alterations in cell morphology were evident within only a few hours of SSP treatment as illustrated in the phase image in Figure 1A. Using fluorescent images, we positively identified cells containing fluorescently stained DNA indicating activation of caspase-3/7, cleavage of the DEVD moiety in the kinetic apoptosis reagent, and fluorescent labeling of cellular DNA (green image in Figure 1A). Using the object counting algorithm, we successfully quantified the number of

fluorescent objects as indicated with red x's in Figure 1A. The object counting criteria were then applied to all images in the experiment at each time point. The data in Figure 1B indicate that caspase-3/7 activation is detectable within a few hours of SSP treatment, with a maximal response triggered in the presence of 333 nM SSP. As expected, increasing amounts of SSP not only triggered apoptosis in a concentration dependent manner, but also dramatically altered cell proliferation. To account for this observation, we also completed an end point analysis at the 48 hour time point. To do so, the Vybrant DyeCycle Green DNA dye was added directly (no wash required) to the wells at a final concentration of 1 μ M in 50 μ l of PBS. After a 30 minute incubation, the total number of DNA containing objects was enumerated using the object counting algorithm. As expected, our data indicate an inverse correlation between the total number of objects and the apoptotic index as a function of increasing concentrations of SSP (Figure 1C). The data clearly indicate the advantage of seeing all the kinetic time points as they occur, thereby alleviating the need to pick an end-point for analysis *a-priori* to running the experiment.

The Essen CellPlayer™ Kinetic Caspase-3/7 Apoptosis reagent specifically measures the activation of caspase-3/7.

It is well known that addition of SSP can trigger apoptosis through multiple pathways. Therefore, we sought to verify that the kinetic apoptosis reagent was specific to caspase-3/7, and not to other caspases. It has been demonstrated that the breast carcinoma cell line, MCF-7, lacks caspase-3 expression[6]. This is a result of a 47-base pair deletion within exon 3 of the *CASP-3* gene resulting in altered mRNA splicing and the introduction of a premature stop codon that halts translation of the *CASP-3* mRNA. Early studies showed that although MCF-7 cells remain sensitive to both SSP and TNF α induced apoptosis, morphological alterations including shrinkage, blebbing and DNA fragmentation were not observed suggesting a critical role for caspase-3 in mediating these apoptosis specific characteristics. Therefore, MCF-7 cells are an ideal model cell strain to determine whether caspase-3 is required for positive identification of apoptotic cells using this reagent.

Following treatment with a mid-range concentration of 100 nM SSP, we did not observe caspase-3/7 activation in MCF-7 cells at any time point tested (Figure 2). In contrast, a significant induction of caspase-3/7 activity was detectable in identically treated MDA-MB-231 cells. Endpoint calculations of percent apoptosis indicated a slight induction of caspase-3/7

activation in 100 nM SSP treated MCF-7 cells. It is possible that caspase-7, another executioner caspase that cleaves at the DEVD motif, is responsible for this activity. Nevertheless,

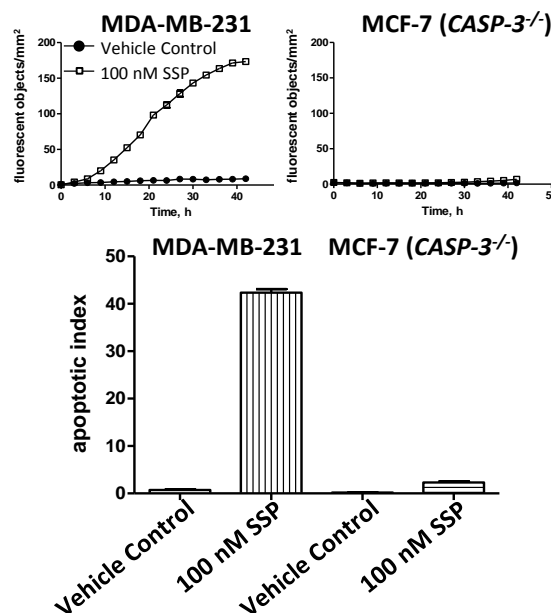


Figure 2: The Essen CellPlayer™ Kinetic Caspases-3/7 Apoptosis reagent requires caspase-3 for detection of apoptotic nuclei. SSP induced caspase-3/7 activity in MDA-MB-231 cells (*CASP-3^{+/+}*) was compared to MCF-7 cells (*CASP-3^{-/-}*). The number of fluorescent objects, correlative to caspase-3/7 activity, was determined using the IncuCyte™ object counting algorithm. The apoptotic index was calculated by dividing the number of caspase-3/7 fluorescent objects by the total number of DNA containing objects following staining with Vybrant DyeCycle Green.

these data clearly illustrate that the kinetic apoptosis reagent can be utilized to measure caspase-3/7 activation induced by SSP, and that caspase-3 is required for positive identification of apoptotic cells using this reagent.

Using Images and Movies to Confirm Signaling

One of the major advantages of using the IncuCyte™ FLR is the ability to verify the quantified kinetic data with both phase contrast and fluorescent images. Classical morphological changes associated with apoptosis include: cell shrinkage, membrane blebbing, nuclear condensation, and DNA fragmentation. The time lapse sequence presented in Figure 3 clearly highlights this advantage, illustrating the ability to use phase contrast and fluorescent blended images to temporally correlate the activation of caspase-3/7 with morphological changes in response to treatment with Taxol. Using IncuCyte™ FLR, the temporal responses in every well can be



supplemented with a “movie” of either a phase contrast, fluorescence or blended time-lapse sequence. This ability significantly enhances the confidence in the measured response and any subsequent conclusions drawn from quantitative image analysis.

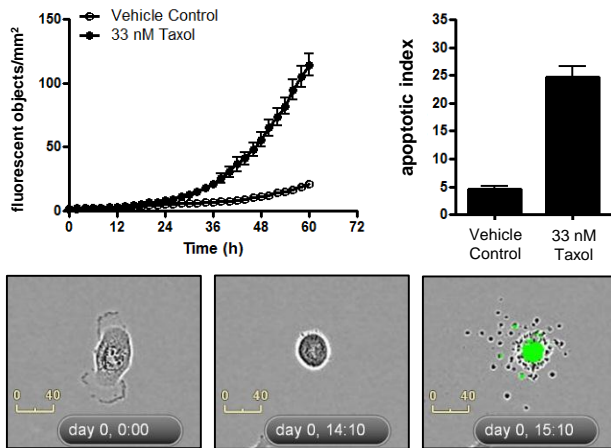


Figure 3: Taxol induced caspase-3/7 mediated apoptosis in human breast adenocarcinoma cells. MDA-MB-231 cells were treated with 33 nM taxol and the number of caspase-3/7 positive cells was quantified using the IncuCyte™ object counting algorithm. The apoptotic index was calculated by dividing the number of caspase-3/7 fluorescent objects by the total number of DNA containing objects following staining with Vybrant DyeCycle Green. **Bottom panels**, classical morphological changes associated with apoptotic cells were observed using phase contrast images

Validation of the Essen CellPlayer™ Kinetic Apoptosis Assay

Multiple factors must be considered when choosing an assay. These factors include, but are not limited to: 1) the statistical reproducibility of the assay, 2) assay throughput, 3) the cost including time, labor and reagents, 4) the added information content, e.g. endpoint vs. kinetic, and 5) assay preparation considerations, e.g. no-wash vs. multiple wash labeling. To assess the statistical reproducibility of the assay, we completed a series of experiments using multiple cell types and control compounds. These experiments included measuring apoptosis in both a breast cancer cell line (MDA-MB-231) as well as in primary HUVECs.

Statistical Validation using MDA-MB-231 Cells

In the first series of experiments we sought to demonstrate the statistical reproducibility of this assay using SSP induced apoptosis in MDA-MB-231 cells. Individual wells of a 96-well plate were spiked with three different concentrations of

SSP (5 nM, 25 nM, and 75 nM) in duplicate plates. Images were taken at three hour intervals and the number of fluorescent objects per unit area was plotted on a per-well basis in a 96-well format as illustrated in Figure 4A. These data clearly show that wells receiving low, moderate, and high doses of an apoptosis inducing reagent are clearly discernable from each other both visually and quantitatively.

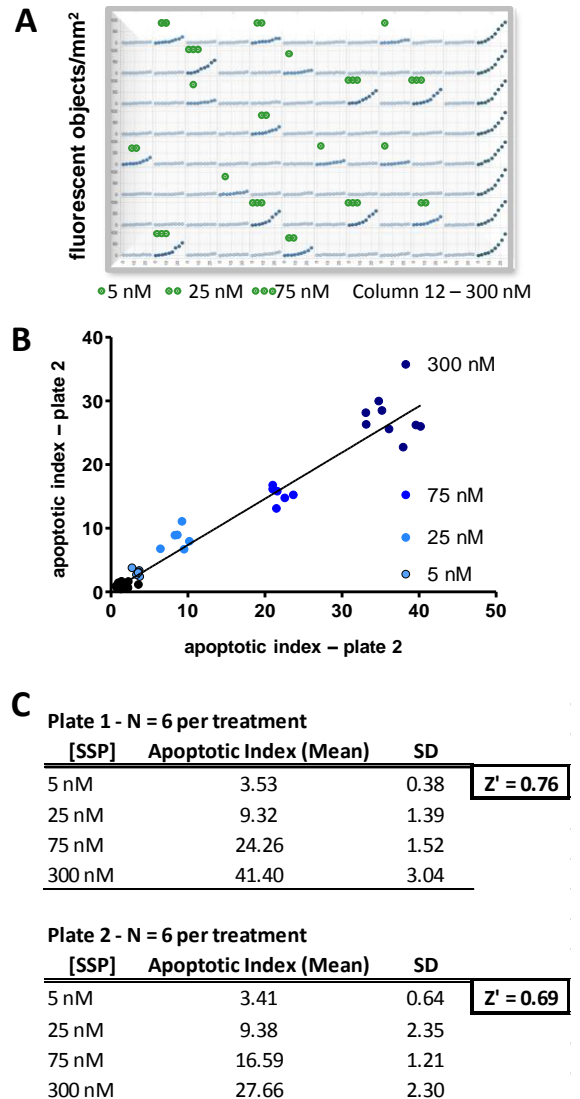


Figure 4: Statistical reproducibility of apoptotic response of MDA-MB-231 cells to SSP. (A) 96-well platemap showing reproducibility of single-well responses to various concentrations of SSP on MDA-MB-231 cells. The CellPlayer™ Apoptosis Assay is amenable to single-shot screening. (B) Linear regression plot comparing two different test plates spiked with the same concentrations of SSP. (C) Statistical measures from the same two plates.



Using these data we completed a number of additional statistical analyses. First, we evaluated the plate to plate reproducibility of the assay by plotting the data from replicate plates on different axes and analyzing the data using linear regression. The resulting R^2 value of 0.97 indicates a strong correlation between identically treated wells on separate plates indicating strong inter-plate reproducibility (Figure 4B). Figure 4C represents several other assay parameters. As indicated by the means and standard deviations of the apoptotic index calculated for individual treatment groups, strong intra-plate reproducibility was also observed. Both plates had Z' values exceeding 0.65[7].

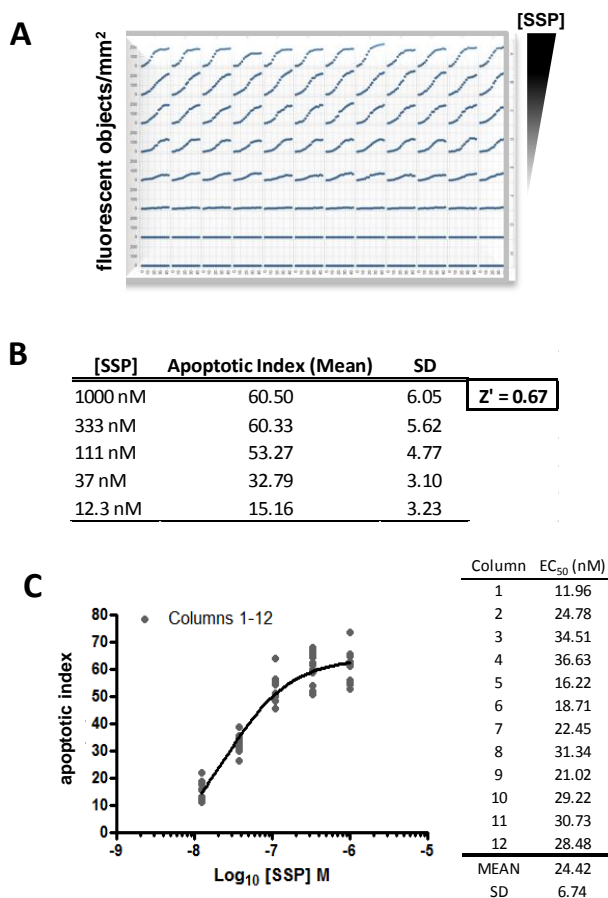


Figure 5: Reproducibility of single plate concentration response of MDA-MB-231 cells to SSP. (A) 96-well platemap showing the reproducibility of concentration response to high (top) and low (bottom) concentrations of SSP. (B) Statistical analysis of the concentration response from rows of plate described in A. (C) Consistency of 12 EC₅₀ determinations from plate described in A.

Importantly, by strategically spiking both edge and interior wells on the microplate with SSP, we were able to statistically determine that well location did not alter the apoptotic response i. e. we did not observe any “edge effects”.

Together, these data indicate that the kinetic apoptosis reagent can be used in the IncuCyte™ FLR to generate statistically robust data using a small number of replicate samples and is amenable to medium throughput assays given the six-plate capacity of the IncuCyte™ FLR platform.

We also evaluated the ability to calculate EC₅₀ values from each column of a 96-well plate. To do this, we treated each row of MDA-MB-231 cells with 3-fold decreasing concentrations of SSP, as illustrated in Figure 5. Again, we observed highly reproducible kinetic inductions of caspase-3/7 activity correlating to decreasing concentrations of SSP. Using the calculated apoptotic index, we also show how these data can be used to generate EC₅₀ values. The resulting data revealed a very narrow range of calculated EC₅₀ values with an excellent Z' -factor of 0.67. Since the IncuCyte™ FLR is capable of holding six 96-well assay plates, 576 wells (72, 8-pt concentration response) of kinetic data can be obtained from one experimental trial.

Measuring Apoptosis in Primary HUVECs

In the final experiment, we sought to determine the level of caspase-3/7 mediated apoptosis in primary HUVECs in culture. Low passage HUVECs were plated in media with decreasing levels of serum supplementation and 5 μ M Caspase-3/7 apoptosis reagent, placed in the IncuCyte™ FLR within an incubator, and data was collected over the course of 3 days. As shown in Figure 6, positive identification of cells with activated caspase-3/7 was observed within 20 hours of culture in cells plated in media with less than 2% serum. Interestingly, we also observed a significant amount of caspase-3/7 positive cells in both media supplemented with 4% serum, and in complete media arising at approximately 25-30 hours post assay initiation. Using endpoint data, we found that nearly 5-10% of HUVECs cultured in complete media were apoptotic. These data illustrate another utility for this reagent, specifically for use in cell culture quality control experiments.



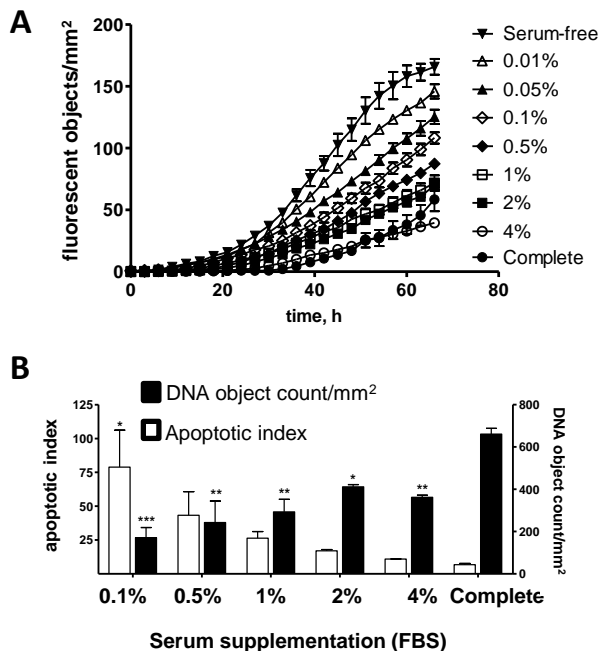


Figure 6: Apoptotic response of HUVECs to serum deprivation. (A) Kinetic measures of the number of caspase-3/7 positive cells is recorded over time and plotted as fluorescent objects, n=3 wells per data point shown (C) At the 62 hour end point, the apoptotic index was calculated by dividing the number of caspase-3/7 fluorescent objects by the total number of DNA containing objects following staining with Vybrant DyeCycle Green.

Conclusions

This application note demonstrates the following attributes of the Essen BioScience 96-Well CellPlayer™ Kinetic Caspase-3/7 Apoptosis assay.

- 1) The apoptotic signal relies on activation of Caspase-3/7, a primary and irreversible “executioner” pathway in most cell types.
- 2) The assay format follows a homogeneous “mix and read” protocol which can be run over multiple days in full media. There are no wash or lifting steps required, negating the concern that cells are lost during the experiment or labeling process.
- 3) The assay provides a full kinetic readout of apoptotic signaling over multiple days from within your cell culture incubator. Aside from providing insight into the dynamics and timing of the apoptotic signaling pathway, this attribute eliminates the need for determining a single, optimum, assay endpoint *a-priori*; something which can vary considerably for different cell types and for different compound treatment conditions.

- 4) The assay has high statistical reproducibility and can be used both for single-point screening or concentration response profiling. Given the six 96-well plate capacity of the IncuCyte™ FLR, up to 576 wells, or 72, 8-pt response curves can be acquired on the same experiment.
- 5) All data points and temporal data curves can be subsequently validated by individual images or time-lapse movies respectively. The kinetic readout of the IncuCyte™ FLR provides both high contrast (HD) phase as well as quantitative fluorescent imaging. These data can be used to validate and confirm the integrated image processing metrics provided in the IncuCyte™ FLR analysis package.

Together, these attributes provide a new and unique assay for apoptotic pathway analysis for both drug discovery as well as basic cell biology research.





References

1. Cotter TG: *Apoptosis and Cancer: The Genesis of a Research Field*. *Nat Rev Cancer* 2009, **9**(7):501-507.
2. Shi Y: *Mechanisms of Caspase Activation and Inhibition During Apoptosis*. *Mol Cell* 2002, **9**(3):459-470.
3. Thornberry NA, Rano TA, Peterson EP, Rasper DM, Timkey T, Garcia-Calvo M, Houtzager VM, Nordstrom PA, Roy S, Vaillancourt JP, Chapman KT, Nicholson DW: *A Combinatorial Approach Defines Specificities of Members of the Caspase Family and Granzyme B. Functional Relationships Established for Key Mediators of Apoptosis*. *J Biol Chem* 1997, **272**(29):17907-17911.
4. Daya S, Robets M, Isherwood B, Ingleston-Orme A, Caie P, Teobald I, Eagle R, Carragher N: *Integrating an Automated in Vitro Combination Screening Platform with Live-Cell and Endpoint Phenotypic Assays to Support the Testing of Drug Combinations*. *SBS 16th Annual Conference and Exhibition* 2010.
5. Cen H, Mao F, Aronchik I, Fuentes RJ, Firestone GL: *Devd-Nucview488: A Novel Class of Enzyme Substrates for Real-Time Detection of Caspase-3 Activity in Live Cells*. *FASEB J* 2008, **22**(7):2243-2252.
6. Janicke RU, Sprengart ML, Wati MR, Porter AG: *Caspase-3 Is Required for DNA Fragmentation and Morphological Changes Associated with Apoptosis*. *J Biol Chem* 1998, **273**(16):9357-9360.
7. Zhang JH, Chung TD, Oldenburg KR: *A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays*. *J Biomol Screen* 1999, **4**(2):67-73.

About the IncuCyte™ Live-Cell Imaging System

The Essen BioScience IncuCyte™ Live-Cell Imaging System is a compact, automated microscope. The IncuCyte™ resides inside your standard tissue culture incubator and is used for long-term kinetic imaging. To request more information about the IncuCyte™, please visit us at www.essenbioscience.com.

