

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

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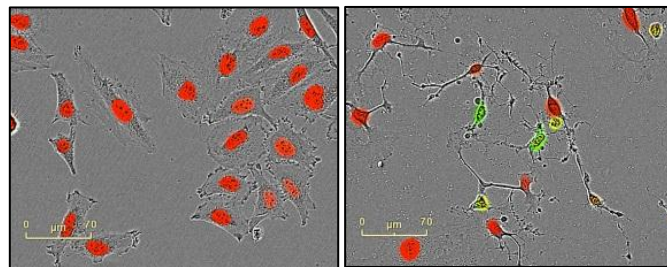
## Introduction

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 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 (cysteiny 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™ or ZOOM™ imaging systems. 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]. In addition, Essen's lentivirus based CellPlayer NucLight Red reagent, used to reliably label cell nuclei in a nonperturbing way, provides a means to kinetically quantify cell proliferation over time (Figure 1). In combination, the caspase-3/7 reagent and NucLight label provide a multiplexed way to differentiate inhibition of cell growth and induction of cell death. High definition phase contrast images provide an additional qualitative validation of cell death based on morphological characteristics. Finally, using any number of strategies (e.g. area under the curve, max counts, single time points), the kinetic data generated using this assay strategy can be used to derive informed pharmacology measurements.



**Figure 1: HeLa cells expressing NucLight Red.** (Left) Untreated HeLa NucLight Red cells. (Right) HeLa NucLight Red cells treated with 300 nM staurosporine. Blended phase-contrast and red/green images taken at 20x illustrate homogenous express of red nuclear signal and activation of caspase-3/7 (green). HD phase images provide a qualitative assessment of morphological correlates to apoptosis (left).

## Approach and Methods

### Cell culture and assay procedure

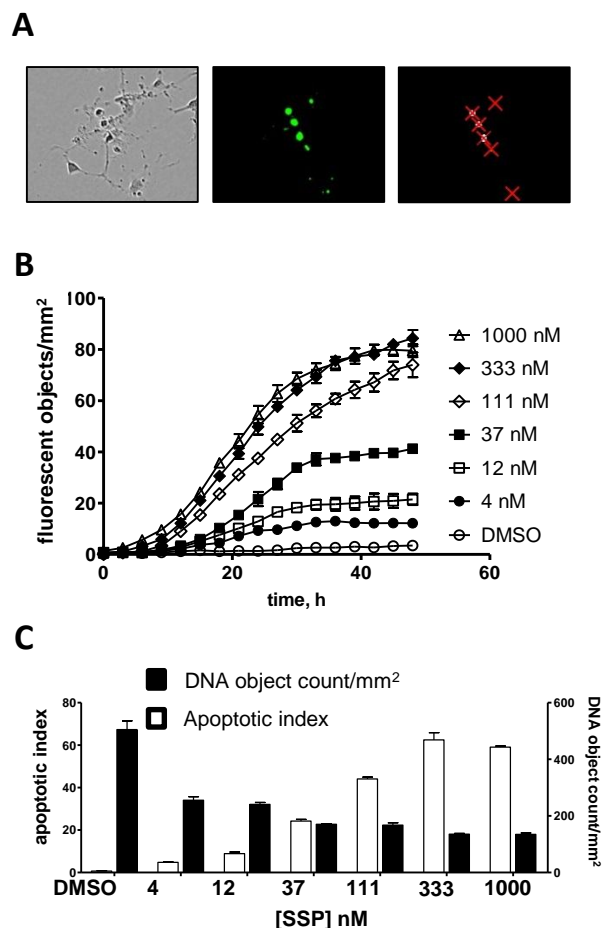
Prior to beginning the assay, cells were grown to confluence in 25 cm<sup>2</sup> tissue culture-treated flasks. MDA-MB-231, HeLa, A549, and MCF-7 cells were cultured in F12-K (Gibco)



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. HeLa cells were infected with Essen's CellPlayer NucLight Red (Lenti, EF1a, puromycin) reagent (MOI of 3 TU/cell). Positively expressing cells were selected for in complete media containing 1 µg/ml puromycin for 24 hours and then maintained in 0.5 µg/ml puromycin. The day before starting the assay, cells were plated at 2500 cells/well (HUVEC) or 5000 cells/well (MDA-MB-231, HeLa NucLight Red, A549, 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. Staurosporine (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. Transcription necrosis factor alpha (TNF-α) was serially diluted in F12-K growth medium (100 µl per well) containing 5 µg/ml cycloheximide and 5 µM kinetic apoptosis reagent. 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 or ZOOM with a 10X objective in a standard cell culture incubator at 37°C and 5% CO<sub>2</sub>. 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

#### 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. As an additional marker of proliferation, and to correct for differential proliferation of cells in one color (green) experiments, the total number of DNA containing objects was counted at the final time point using Vybrant



**Figure 2: Staurosporine (SSP) induced caspase-3/7 activity in human breast adenocarcinoma cells (MDA-MB-231).** (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 (IncuCyte FLR). (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.

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 and ZOOM*

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



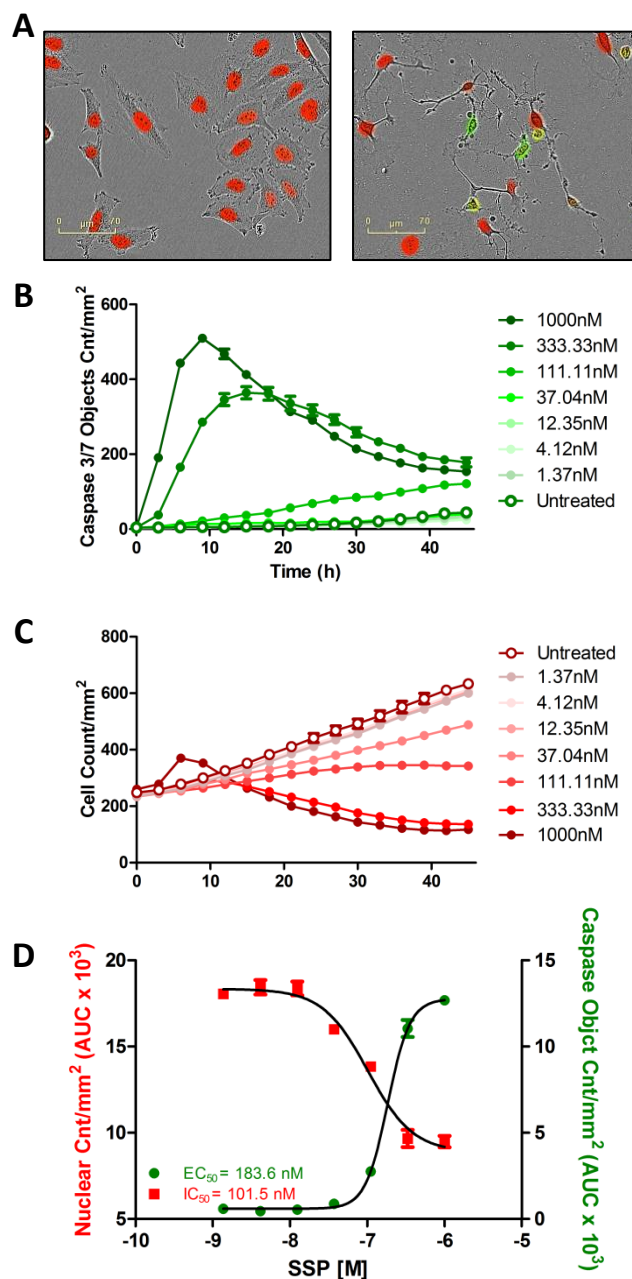
general protein kinase inhibitor staurosporine (SSP). To accomplish this, we treated MDA-MB-231 cells, a human breast adenocarcinoma derived cell line with SSP serially diluted in growth media containing 5  $\mu\text{M}$  kinetic apoptosis reagent in a 96-well plate. Once treated, the cells were immediately placed inside the IncuCyte FLR imaging platform with a 10X objective in a standard cell culture incubator and both phase-contrast and fluorescent images were collected every 2-3 hours.

Alterations in cell morphology were evident within only a few hours of SSP treatment as illustrated in the phase image in Figure 2A. 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 2A). Using the object counting algorithm, we successfully quantified the number of fluorescent objects as indicated with red x's in Figure 2A. The object counting criteria were then applied to all images in the experiment at each time point. The data in Figure 2B 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.

Increasing concentrations of SSP also significantly affected cell proliferation. To demonstrate this on the IncuCyte FLR, we completed an end point analysis at the 48 hour time point. Vybrant DyeCycle Green DNA dye was added directly (no wash required) to the wells at a final concentration of 1  $\mu\text{M}$  in 50  $\mu\text{l}$  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 2C). 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.

#### *Multiplexed, kinetic measurements of proliferation and apoptosis*

The two fluorescent channels available on the IncuCyte ZOOM provides a way to kinetically measure caspase-3/7 activation in addition to proliferation (nuclear label) within the same well, thus eliminating the need for end-point analysis. In the next experiment, HeLa NucLight Red cells were treated with SSP in the presence of the 5 $\mu\text{M}$  caspase 3/7 reagent and phase-contrast, red, and green images were collected every 2 hours in IncuCyte ZOOM using a 10x objective (Figure 3; Supplemental Figure 1 - 4x data). These data illustrate typical



**Figure 3: Pharmacological analysis of caspase-3/7 activation and nuclear counts in HeLa NucLight Red cells treated with SSP.** (A) Blended phase-contrast and red/green images taken at 20x show red nuclear signal and activation of caspase-3/7 as well as morphological differences in untreated cells (left) vs. cells treated with 300nM SSP (right). (B) Caspase-3/7 positive objects and (C) nuclear counts were measured over time in response to increasing concentrations of SSP. (D) Area under the curve (AUC) of nuclear counts/mm<sup>2</sup> and caspase object counts/mm<sup>2</sup> over time were measured and used to calculate IC<sub>50</sub> and EC<sub>50</sub> values, respectively.





results obtained using the caspase 3/7 reagent multiplexed with NucLight Red cells to measure the kinetic induction of apoptosis and proliferative effects of drug treatment (Figure 3B and 3C, respectively). Using all of the kinetic data in Figure 3B and 3C, area under the curve (AUC) values were plotted and EC<sub>50</sub> (apoptosis) and IC<sub>50</sub> (proliferation) values were calculated. This 2-color kinetic assay provides a multiplex way to analyze the apoptotic and anti-proliferative effects of various treatments.

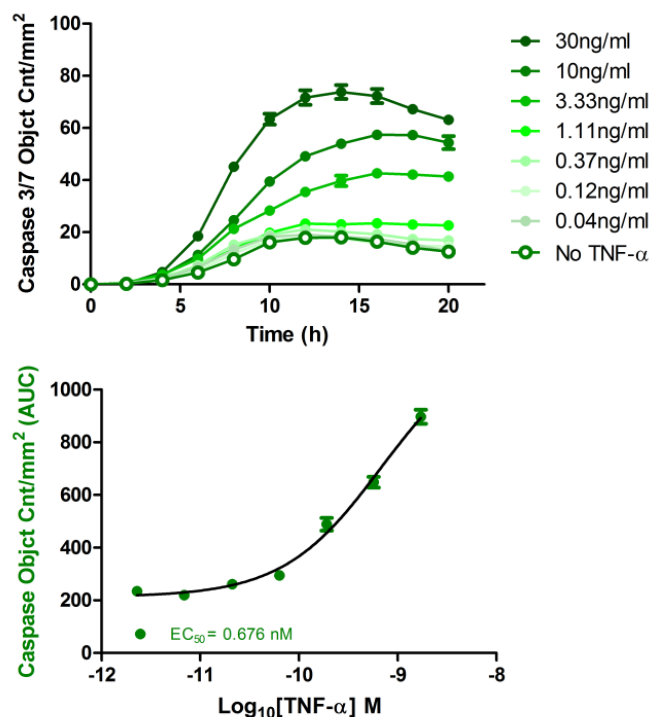
#### *Caspase-3/7 reagent kinetically measures extrinsic activation of apoptosis*

Depending on the cellular context, exposure of cells to Transcription Necrosis Factor alpha (TNF- $\alpha$ ) can induce either pro-survival, or cell death pathways. When used in isolation, TNF- $\alpha$  induces NF $\kappa$ B activity and subsequent expression of pro-survival signaling molecules (e.g. FLIP, XIAP, A20). Alternatively, when used in conjunction with cycloheximide (CHX), itself an inhibitor of translation, TNF- $\alpha$  is a potent inducer of apoptosis through caspase 3 mediated signaling pathways. To demonstrate this using IncuCyte ZOOM, A549

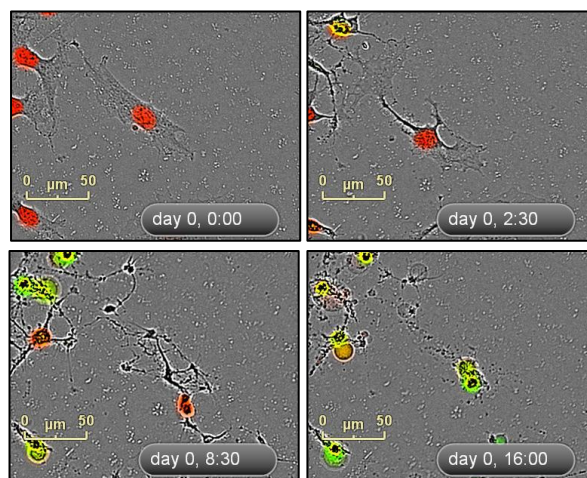
epithelial carcinoma cells were treated with increasing concentrations of TNF- $\alpha$  in the presence of 5 $\mu$ g/ml CHX. Caspase-3/7 was activated in a TNF- $\alpha$  concentration dependent manner (Figure 4A). The AUC values for caspase-3/7 positive objects over time were then used to calculate the EC<sub>50</sub> value of 0.676 nM TNF- $\alpha$  (Figure 4B). Supplemental data shows other examples of apoptosis assays tested on the IncuCyte FLR and ZOOM, such as varying FBS concentrations and the addition of taxol (Supplemental Figures 2 and 3). These data demonstrate the ability to test a variety of apoptotic inducing conditions on many cell types using this kinetic, multiplexed assay.

#### *Using Images and Movies to Confirm Signaling*

One of the major advantages of using the IncuCyte 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 5 highlights this advantage, illustrating the ability to use phase contrast and fluorescent blended images to temporally correlate the activation of caspase-3/7 and the loss of red nuclei due to cell death with morphological changes in response to treatment with SSP. Using the IncuCyte 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



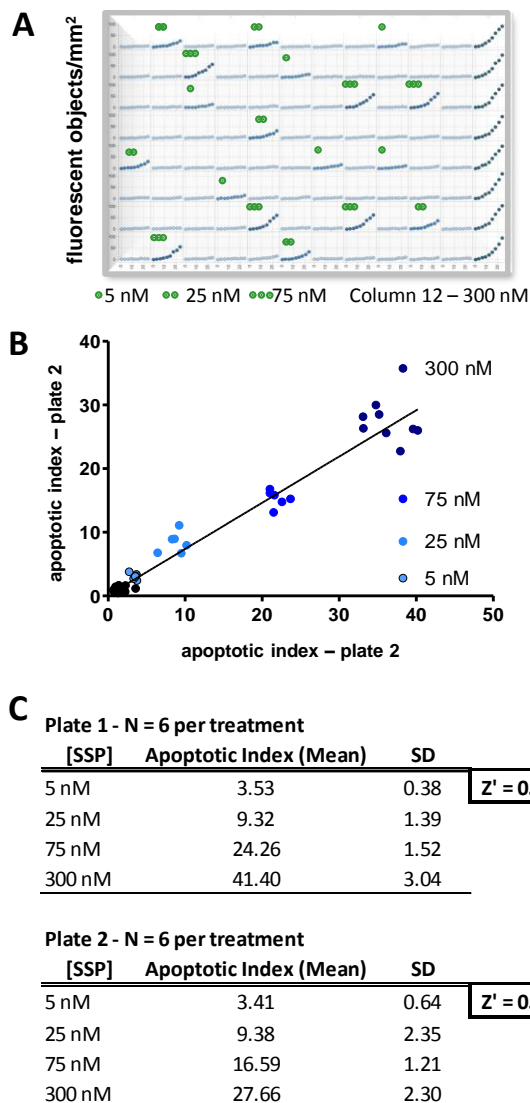
**Figure 4: Extrinsic activation of caspase-3/7 in A549 lung epithelial cells.** (A) Caspase-3/7 activation of A549 lung epithelial cells in response to varying concentrations of TNF- $\alpha$  in the presence of 5 $\mu$ g/ml cycloheximide (CHX). (B) The EC<sub>50</sub> value of TNF- $\alpha$  was calculated by using the area under the curve (AUC) of caspase-3/7 objects/mm<sup>2</sup> over time.



**Figure 5: Time-lapse images and movies to detect SSP induced apoptosis in HeLa cells.** HeLa NucLight Red cells were treated with 300nM SSP in the presence of 5 $\mu$ M Caspase-3/7 reagent and imaged in IncuCyte ZOOM every 30 minutes. Time-lapse images and movies monitor changes in morphology and confirm the activation of the green caspase-3/7 signal in Figure 2B and the loss of the red nuclear signal in Figure 2C.



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



**Figure 6: 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.

subsequent conclusions drawn from quantitative image analysis. See Supplemental Figure 3 for time-lapse images corresponding to data collected on an FLR.

### 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

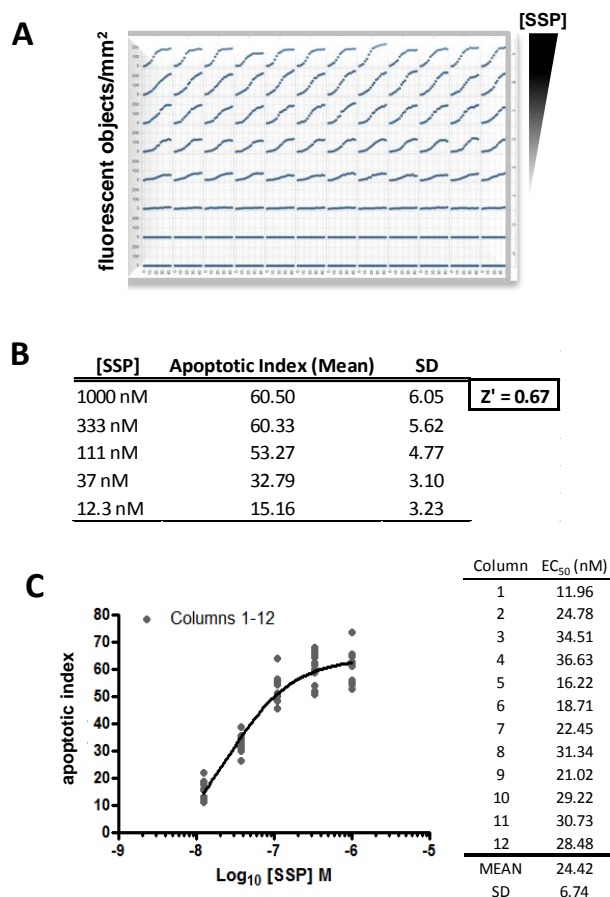
In the first series of experiments we sought to demonstrate the statistical reproducibility of this assay using SSP induced apoptosis in MDA-MB-231 cells. Individual wells of a 96-well plate were spiked with three different concentrations of SSP (5 nM, 25 nM, and 75 nM) in duplicate plates. Images were 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 6A. These data clearly show that wells receiving low, moderate, and high doses of an apoptosis inducing reagent are clearly discernible from each other both visually and quantitatively.

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 6B). Figure 6C 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]

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 7. 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





**Figure 7: 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 EC<sub>50</sub> determinations from plate described in A.

can be used to generate EC<sub>50</sub> values. The resulting data revealed a very narrow range of calculated EC<sub>50</sub> values with an excellent Z-factor of 0.67. Since the IncuCyte FLR is capable of holding six 96-well or 384-well assay plates, 576-2304 wells (72-144, 8-pt concentration response) of kinetic data can be obtained from one experimental trial.

## 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 (see Supplemental Figure 4 for specificity data).
- 2) Cells can be labeled with NucLight reagents to measure cell proliferation and kinetically monitor anti-proliferative

effects of compounds in addition to activation of Caspase-3/7

- 3) IC<sub>50</sub> and EC<sub>50</sub> values can be calculated using kinetic area under the curve values of nuclear counts and caspase-3/7 counts, respectively.
- 4) 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.
- 5) 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.
- 6) 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 and ZOOM, up to 576 wells, or 72, 8-pt response curves can be acquired on the same experiment.
- 7) 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 and ZOOM 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 and ZOOM 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

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### 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](http://www.essenbioscience.com).







## NOTES

