

Kinetic measurement of apoptosis using CellPlayer™ 96-Well Caspase-3/7 reagent on the IncuCyte™ FLR

Apoptosis is an active process by which cells undergo programmed cell death and is required for development, tissue homeostasis, and immunity. Both intrinsic and extrinsic factors lead to activation of the apoptotic program, which in most cases converges on the enzyme caspase-3. In a normal, healthy cell, caspase-3 exists as an inactive zymogen. Following an apoptotic stimulus, initiator caspases cleave caspase-3 into its active form. Once activated, this executioner caspase is capable of cleaving a variety of effector substrates at a specific 4 amino acid recognition motif (DEVD) resulting in their activation and leading to disassembly of the cell cytoskeleton, and enzymatic digestion of nuclear DNA.

The Essen BioScience CellPlayer 96-Well Kinetic Caspase-3/7 reagent couples the caspase-3/7 recognition motif (DEVD) to a DNA intercalating dye. This reagent, specially formulated for use in the Essen BioScience IncuCyte FLR, can be added directly to tissue culture wells using a no-wash, mix-and-read protocol to acquire live cell images of cells undergoing caspase-3/7 mediated apoptosis. Addition of this reagent is non-perturbing to cell growth and morphology, and enables the kinetic detection of caspase-3/7 activity using the IncuCyte FLR live cell imaging system while never leaving the incubator. When added to tissue culture medium, this inert, non-fluorescent substrate crosses the cell membrane where it is cleaved by activated caspase-3/7 resulting in the release of the DNA dye and green fluorescent staining of nuclear DNA. Kinetic activation of caspase-3/7 can be monitored morphologically using live cell imaging, and quantified using the IncuCyte FLR object counting algorithm.

Sample Protocol:

Day 0:

- 1) Plate 2×10^3 - 1×10^4 cells per well in a 96-well plate such that the next day, cells are approximately 10-20% confluent (N=3 wells per treatment is recommended). For example, 2.5×10^3 HT-1080 and MDA-MB-231 cells are 10-20% confluent 12-18 hours post seeding. Confluence can be monitored in the IncuCyte FLR.

Day 1:

- 2) Treatment preparation
 - a. Dilute caspase-3/7 reagent to a final concentration of 5 μ M in desired medium formulation. This equates to a 1:1000 dilution of stock reagent. We recommend medium with low levels of riboflavin to reduce the fluorescence background. EBM, F12-K, and Eagles MEM have low riboflavin (<0.2 mg/L). DMEM and RPMI have high riboflavin (>0.2 mg/L).

NOTE: All test agents will be diluted in this medium, so make up a volume that will accommodate all treatment conditions and reagent dilutions.



- b. A volume of 100 µl per well is generally sufficient for the duration of the assay.
- 3) Add prepared treatments to cells
- 4) Place the plate within a microplate tray into the IncuCyte FLR
- 5) Set Scan Type to “Fluorescence & Phase-Contrast”
- 6) Acquire images every 2-3 hours. At least 2 images per well is recommended
NOTE: A delay of 10-15 minutes before the first scan is recommended to allow the plate sufficient time to equilibrate to the incubator environment. Insufficient equilibration may result in condensation on the bottom of lid compromising image quality.

Ending the assay and data analysis

Assay duration will vary depending on the apoptotic stimulus and cell type used. It is often helpful to complete an object counting analysis on a small subset of wells every 24 hours to track the experiment’s progress. However, the IncuCyte FLR will continue to collect and store data until the plate is removed from the instrument, and therefore the “end” of the assay may be determined retroactively. Data analysis is best done using the object counting analysis built into the IncuCyte FLR software, although the specific metric used to measure caspase-3/7 activation is up to the user. Common metrics include both object count/mm² and object confluence. For more complete information, see the CellPlayer 96-Well Caspase-3/7 Apoptosis Application Note at <http://www.essenbioscience.com/productsAppApoptosis.html>.

Optional: Endpoint Normalization

It is often common practice to normalize the number of caspase-3/7 positive objects at the end of the assay to the total number of DNA containing objects in order to factor cell proliferation into the final analysis. We recommend using the Vybrant DyeCycle Green DNA stain available from Life Technologies (<http://products.invitrogen.com/ivgn/product/V35004>) to label all DNA-containing objects at the end of the assay. This stain can be added directly to the wells without aspiration or washing of the caspase-3/7 reagent-containing medium. The final total DNA-containing object count can then be utilized to normalize the data.

Sample Protocol:

- 1) Prepare Vybrant DyeCycle Green stain for end point labeling
 - a. Final concentration of Vybrant DyeCycle Green stain within each well should be 1 µM
 - b. Dilutions of Vybrant DyeCycle Green stain can be made in either culture medium or PBS prior to addition to the wells



**CellPlayer™ 96-Well Kinetic Caspase-3/7 Apoptosis Protocol**

- 2) Add diluted Vybrant DyeCycle Green stain directly to the well immediately after the final caspase-3/7 scan.
NOTE: Removal of existing media is NOT required prior to addition of the Vybrant DyeCycle Green stain.
- 3) Set the plate within a microplate tray into the IncuCyte FLR
- 4) Incubate for 1 hour prior to acquiring final “Fluorescence & Phase-Contrast” images
- 5) Perform Object Counting analysis
- 6) Export the object count data collected during the final scan of the caspase-3/7 assay and paste it into a 3rd party spreadsheet program.
- 7) Export the object count data collected following the staining of objects with Vybrant DyeCycle green.

Calculate Apoptotic Index:

$$\text{Apoptotic Index} = \frac{\# \text{ Caspase} - 3/7 \text{ positive objects}}{\text{Total \# of DNA containing objects}}$$

About the IncuCyte FLR Live-Cell Imaging System:

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

Ordering Information:

CellPlayer 96-Well Kinetic Caspase-3/7 Apoptosis Assay Kit: Contains one vial (20 µl) of 5 mM mix and read reagent in DMSO capable of performing 100-200 tests in 96-well format on the IncuCyte platform.

Part Number: 4440
Price: \$283.50 USD

Orders can be placed at: orders@essenbio.com
Or contact us at: (734) 769-1600

