

Kinetic measurement of cytotoxicity using YOYO[®]-1 iodide reagent on the IncuCyte[™] FLR

Commonly used cytotoxicity assays evaluate a range of end-point parameters, such as the release of lactate dehydrogenase (LDH) and glutathione (GSH) that occur when the cell membrane loses its integrity, ruptures and spills intracellular proteins into the cell culture media. Other methods of measuring cell membrane integrity include the use of propidium iodide and other vital dyes for use in either flow cytometry protocols or fluorescence microscopy. The CellPlayer[™] cytotoxicity assay described in this protocol utilizes YOYO[®]-1 (Life Technologies, Cat #: Y3601) as a cell impermeant cyanine dimer nucleic acid stain that binds to dsDNA which allows for the kinetic evaluation of cytotoxicity using the IncuCyte[™] live-cell imaging system. This membrane-integrity based cytotoxicity assay measures the uptake of a fluorescent dye, normally excluded from intact cells, using kinetic, high resolution imaging. YOYO[®]-1 can be added directly to tissue culture wells using a no-wash, mix-and-read protocol in complete growth medium to acquire live cell images enabling the temporal quantification of cell death. When added to the culture medium, YOYO[®]-1 fluorescently stains the nuclear DNA of cells that have lost plasma membrane integrity and can be monitored morphologically 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 YOYO[®]-1 iodide reagent to a final concentration of 50-100 nM* in desired medium formulation**. This equates to a 1:10,000 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: We recommend titrating the YOYO[®]-1 iodide reagent with your specific cell line and medium in order to determine the optimal YOYO[®]-1 concentration. YOYO[®]-1 iodide should not affect the proliferation rate of the cells.*

***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 cytotoxic 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 cytotoxicity is up to the user. Common metrics include both object count/mm² and object confluence.

Optional: Endpoint Normalization

In order to factor cell proliferation into the final analysis, we recommend normalizing the number of YOYO®-1 positive objects at the end of the assay to the total number of DNA containing objects. We recommend permeablizing the intact cells with the direct addition of 0.0625% Triton X-100 to label all DNA-containing objects with YOYO®-1 at the end of the assay.* The final total DNA-containing object count can then be utilized to normalize the data.

**NOTE: The concentration of Triton X-100 or the incubation time allowed to permeablize cells may have to be adjusted depending upon cell type used.*

Sample Protocol:

- 1) Prepare Triton X-100 for end point labeling
 - a. Final concentration of Triton X-100 within each well should be 0.0625%
 - b. Dilutions of Triton X-100 can be made in either culture medium or PBS prior to addition to the wells
- 2) Add diluted Triton X-100 directly to the wells immediately after the final YOYO-1 scan.

NOTE: Do not remove media from wells. The YOYO®-1 stain present in the treatment is required for assessing total number of DNA containing objects.
- 3) Set the plate within a microplate tray into the IncuCyte™ FLR
- 4) Incubate for 15 minutes prior to acquiring final “Fluorescence & Phase-Contrast” images





CellPlayer™ 96-Well Kinetic Cytotoxicity Protocol

- 5) Perform Object Counting analysis
- 6) Export the object count data collected during the final scan of the YOYO®-1 assay and paste it into a 3rd party spreadsheet program
- 7) Export the object count data collected following the treatment of wells with Triton X-100

Calculate Cytotoxic Index:

$$\text{Cytotoxic Index} = \frac{\#YOYO - 1 \text{ positive objects}}{\text{Total \# of DNA containing objects}}$$

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.

