

# IncuCyte<sup>®</sup> Cytotoxicity Assay

For the fluorescent quantification of cell death

This protocol provides an overview of the IncuCyte<sup>®</sup> Cytotoxicity Assay methodology which uses the mix-and-read IncuCyte<sup>®</sup> Green or Red Reagent to detect cell death in real time. The protocol is compatible with the IncuCyte<sup>®</sup> live-cell analysis system and your choice of cells (e.g., tumor, immune, neuronal) and treatments.

Furthermore, this protocol can be used with cells labeled using the IncuCyte<sup>®</sup> NuLight nuclear labeling reagents to provide multiplexed measurements of proliferation alongside cell death in the same well.

## Required materials

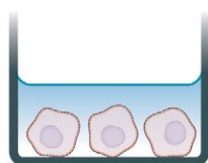
- IncuCyte<sup>®</sup> Red Cytotoxicity Reagent (EssenBioscience Cat #4632)  
*or*
- IncuCyte<sup>®</sup> Green Cytotoxicity Reagent (EssenBioscience Cat #4633)
- Poly-L-ornithine (Sigma P4957)  
– optional, for non-adherent cells
- Fibronectin (Sigma A7906)  
– optional, for non-adherent cells
- Flat bottom tissue culture plate (e.g., Corning 3595, TPP 92096 for neuronal cell health)

## General Guidelines

- We recommend medium with low levels of riboflavin to reduce the green 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).
- Following cell seeding, place plates at ambient temperature (15 minutes for adherent cell lines and 45 minutes for non-adherent cell lines) to ensure homogenous cell settling.
- Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.
- After placing the plate in the IncuCyte<sup>®</sup> live-cell analysis system, allow the plate to warm to 37 °C for 30 minutes prior to scanning
- If monitoring cytotoxicity in primary neuronal cultures, we recommend use of the IncuCyte<sup>®</sup> Cytotox Red reagent to eliminate risk of green channel excitation issues in these sensitive cell types.

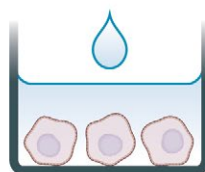
## Adherent Cell Line Protocol

### 1 SEED CELLS



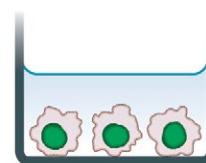
Seed cells (100 µL/well, 1,000 – 5,000) into a 96-well plate and incubate overnight.

### 2 PREPARE CYTOTOXICITY REAGENT AND TREAT CELLS



Prepare the desired treatments at 1x in medium containing IncuCyte Cytotoxicity reagent. Aspirate media from wells and add treatment (100 µL/well).

### 3 LIVE CELL FLUORESCENT ANALYSIS



Capture images every 2-3 hours (20x or 10x) in the IncuCyte<sup>®</sup> System. Analyze using integrated software.

## Day 0:

### 1 Seed effector cells

1.1. Seed your choice of cells (100 µL per well) at an appropriate density into a 96-well plate, such that by day 1 the cell confluence is approximately 30%. The seeding density will need to be optimized for the cell line used; however, we have found that 1,000 to 5,000 cells per well (10,000 – 50,000 cells/mL seeding stock) are reasonable starting points.

**NOTE:** For non-proliferating cell lines (e.g., rate forebrain neurons) we recommend seeding at  $15 \times 10^3$  to  $30 \times 10^3$  cells per well, and culturing for 14 days for the neural network to establish, prior to evaluating cytotoxicity.


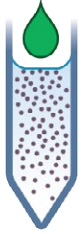
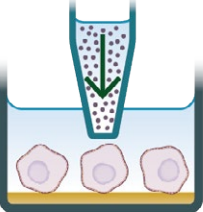
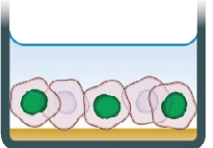
- a. Monitor cell growth using the IncuCyte system to capture phase contrast images every 2 hours and analyze using the integrated confluence algorithm.

Day 1:

- 2 Cytotoxicity reagent preparation and cell treatment addition**
- 2.1. Dilute cytotoxicity reagent in desired medium formulation.  
**NOTE:** All test agents will be diluted in this reagent-containing medium, so make up a volume that will accommodate all treatment conditions. The volumes/dilutions added to cells may be varied; however, a volume of 100  $\mu$ L per well is generally sufficient for the duration of the assay.
- 2.2. Remove the cell plate from the incubator and aspirate off growth medium.
- 2.3. Add treatments and controls to appropriate wells of the 96-well plate.

- 3 Live-cell imaging of cytotoxicity**
- 3.1. Place the cell plate into the IncuCyte Live-Cell Analysis System and allow the plate to warm to 37°C for 30 minutes prior to scanning.
- Objective: 10x or 20x
  - Channel selection: Phase Contrast and Fluorescence
  - Scan type: Standard (2-4 images per well)
  - Scan interval: Typically, every 2 hours, until your experiment is complete.
- NOTE:** For neuronal cultures we recommend scanning every 6 to 12 hours to minimize risk of phototoxicity.

Non-Adherent Cell Line Protocol

<p><b>1 COAT PLATE</b></p>  <p>Coat plate with 0.01% poly-L-ornithine solution or 5 <math>\mu</math>L/mL fibronectin diluted in 0.1% BSA.</p>	<p><b>2 PREPARE INCUCYTE CYTOTOXICITY REAGENT AND TREATMENT</b></p>  <p>Dilute cytotoxicity reagent in medium at 1x and prepare cell treatments.</p>	<p><b>3 SEED CELLS AND ADD TREATMENT</b></p>  <p>Seed cells (100 <math>\mu</math>L/well, 5,000 – 25,000 cells) into the coated 96-well plate. Immediately add cytotoxicity <math>\pm</math> treatments and triturate.</p>	<p><b>4 LIVE CELL FLUORESCENT ANALYSIS</b></p>  <p>Capture images every 2-3 hours (20x or 10x) in the IncuCyte<sup>®</sup> System.</p>
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Day 1:

- 1 Coat Plate**
- 1.1. Coat a 96-well flat bottom plate with appropriate coating matrix. We recommend coating with 50  $\mu$ L of either 0.01% poly-L-ornithine solution (Sigma P4957) or 5  $\mu$ g/mL fibronectin (Sigma A7906) diluted in 0.1% BSA. Coat plates for 1 hour at ambient temperature, remove solution from wells, then allow plates to dry for 30-60 minutes prior to cell addition.
- 2 Prepare cytotoxicity reagent and treatments**
- 2.1. Prior to cell seeding, dilute cytotoxicity reagent to a final concentration of 250 nM (1:4000 dilution) in desired medium formulation.  
**NOTE:** All test agents will be diluted in this reagent-containing medium, so make up a volume that will accommodate all treatment conditions. The volumes/dilutions added to cells may be varied; however, a volume of 100  $\mu$ L per well is generally sufficient for the duration of the assay.
- 2.2. Prepare cell treatments at 2x final assay concentration in enough cell culture medium the cytotoxicity reagent to achieve a volume of 100  $\mu$ L per well.
- 3 Seed cells and add prepared treatments**
- 3.1. Seed your choice of cells (100  $\mu$ L per well) at an appropriate density into a 96-well plate in medium containing the cytotoxicity reagent. The seeding density will need to be optimized for the cell line used; however, we have found that 5,000 to 25,000 cells per well (50,000 – 250,000 cells/mL seeding stock) are reasonable starting points.
- 3.2. Immediately add treatments and controls to appropriate wells of the 96-well plate containing cells. Triturate wells to appropriately mix the treatment to ensure cell exposure at 1x.
- 4 Live-Cell Imaging of cytotoxicity**
- 4.1. Place the cell plate into the IncuCyte<sup>®</sup> live-cell analysis system and allow the plate to warm to 37°C for 30 minutes prior to scanning.
- Objective: 4x (recommended 1 image per well or whole well) or 10x
  - Channel selection: Phase Contrast and Fluorescence
  - Scan type: Standard
  - Scan interval: Typically, every 2 hours, until your experiment is complete.

### Related Products and Applications

A comprehensive range of fluorescent nuclear labeling and cell health reagents are available for use with the IncuCyte® live-cell analysis system to enable multiplexed measurements of apoptosis and proliferation alongside cytotoxicity.

Product	Cat No.	Amount
IncuCyte® NuLight Red BacMam 3.0 Reagent for nuclear labeling	4621	1 mL
IncuCyte® NuLight Green BacMam 3.0 Reagent for nuclear labeling	4622	1 mL
IncuCyte® NuLight Green Lentivirus Reagent (EF-1 $\alpha$ , Puro) for nuclear labeling	4624	0.2 mL
IncuCyte® NuLight Red Lentivirus Reagent (EF-1 $\alpha$ , Puro) for nuclear labeling	4625	0.2 mL
IncuCyte® NuLight Green Lentivirus Reagent (EF-1 $\alpha$ , Bleo) for nuclear labeling	4626	0.2 mL
IncuCyte® NuLight Red Lentivirus Reagent (EF-1 $\alpha$ , Bleo) for nuclear labeling	4627	0.2 mL
IncuCyte® NuLight Green Lentivirus Reagent (EF-1 $\alpha$ , Puro) for nuclear labeling	4475	0.6 mL
IncuCyte® NuLight Red Lentivirus Reagent (EF-1 $\alpha$ , Puro) for nuclear labeling	4476	0.6 mL
IncuCyte® NuLight Green Lentivirus Reagent (EF-1 $\alpha$ , Bleo) for nuclear labeling	4477	0.6 mL
IncuCyte® NuLight Red Lentivirus Reagent (EF-1 $\alpha$ , Bleo) for nuclear labeling	4478	0.6 mL
IncuCyte® Cytotox Red Reagent for counting dead cells	4632	5 $\mu$ L x 5
IncuCyte® Cytotox Green Reagent for counting dead cells	4633	5 $\mu$ L x 5
IncuCyte® Annexin V Red Reagent for apoptosis	4641	100 tests
IncuCyte® Annexin V Green Reagent for apoptosis	4642	100 tests
IncuCyte® Caspase-3/7 Green Reagent for apoptosis	4440	20 $\mu$ L

A complete suite of cell health applications is available to fit your experimental needs. Find more information at [essenbioscience.com/applications](http://essenbioscience.com/applications)

For additional product or technical information, please e-mail us at [AskAScientist@essenbio.com](mailto:AskAScientist@essenbio.com)  
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