**IncuCyte® Cytotoxicity Assay**

For the fluorescent quantification of cell death

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

**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® 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® Cytotox Red reagent to eliminate risk of green channel excitation issues in these sensitive cell types.

**Required materials**

- IncuCyte® Red Cytotoxicity Reagent (EssenBioscience Cat #4632)
- IncuCyte® 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)

**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® System. Analyze using integrated software.

**Day 0:**

1. **Seed effector cells**
   - 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 x 10³ to 30 x 10³ 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.

IncuCyte.com
**Day 1:**

1. **Coat Plate**
   
   1.1. Coat a 96-well flat bottom plate with appropriate coating matrix. We recommend coating with 50 µL of either 0.01% poly-L-ornithine solution (Sigma P4957) or 5 µg/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 µL 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 µL per well.

   

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.

   a. **Objective:** 10x or 20x
   
   b. **Channel selection:** Phase Contrast and Fluorescence
   
   c. **Scan type:** Standard (2-4 images per well)
   
   d. **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.

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**Non-Adherent Cell Line Protocol**

1. **Coat Plate**
   
   Coat plate with 0.01% poly-L-ornithine solution or 5 µL/mL fibronectin diluted in 0.1% BSA.

2. **Prepare IncuCyte cytotoxicity reagent and treatment**
   
   Dilute cytotoxicity reagent in medium at 1x and prepare cell treatments.

3. **Seed cells and add treatment**
   
   Seed cells (100 µL/well, 5,000 – 25,000 cells) into the coated 96-well plate. Immediately add cytotoxicity ± treatments and triturate.

4. **Live Cell fluorescent analysis**
   
   Capture images every 2-3 hours (20x or 10x) in the IncuCyte® System.
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.

<table>
<thead>
<tr>
<th>Product</th>
<th>Cat No.</th>
<th>Amount</th>
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<tbody>
<tr>
<td>IncuCyte® NucLight Red BacMam 3.0 Reagent for nuclear labeling</td>
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<td>IncuCyte® NucLight Green BacMam 3.0 Reagent for nuclear labeling</td>
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<td>IncuCyte® Caspase-3/7 Green Reagent for apoptosis</td>
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A complete suite of cell health applications is available to fit your experimental needs. Find more information at essenbioscience.com/applications

For additional product or technical information, please e-mail us at AskAScientist@essenbio.com
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