Protocol

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® S3 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® NucLight nuclear labeling reagents to provide multiplexed measurements of proliferation alongside cell death in the same well.

Required materials

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

or

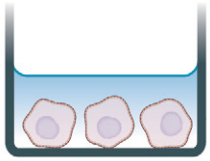
- IncuCyte® Green Cytotoxicity Reagent (Sartorius Cat# 4633)
- Poly-L-ornithine (Sigma P4957)
  - optional, for non-adherent cells
- Fibronectin (Sigma A7906)
  - optional, for non-adherent cells
- Flat bottom tissue culture plate (e.g., Corning 3595, TPP 92096 for neuronal cell health)

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® S3 Live-Cell Analysis System, allow the plate to warm to 37 °C for 30 minutes prior to scanning.
- If monitoring cytotoxcity 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.

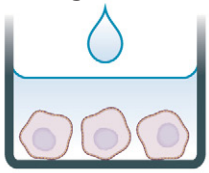
Adherent Cell Line Protocol

1. Seed cells



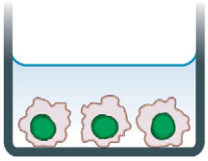
Seed cells (100 µL/well, 1,000 — 5,000) into a 96-well plate and incubate overnight.

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® S3 Live-Cell Analysis 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 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 S3 Live-Cell Analysis 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 µL 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 S3 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.

NOTE: A cytotoxic Index can be calculated on IncuCyte S3 Live-Cell Analysis System using the IncuCyte Cell-by-Cell Analysis Software Module (PN 9600 0031). This enables individual cell identification and subsequent classification into subpopulations based on properties including fluorescence intensity.

a. Scan type: Standard/Adherent Cell-by-Cell

b. Objective: 10x

For further details of this analysis module and it's application see:

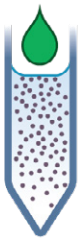
www.essenbioscience.com/cell-by-cell

Non-Adherent Cell Line Protocol

1. Coat plate

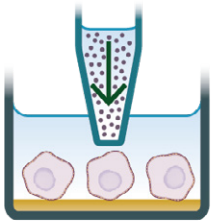
Coat plate with 0.01% poly-L-ornithine solution or 5 µL/mL fibronectin diluted in 0.1% BSA.

2. Prepare Incucyte cytotoxicity reagent and treatment



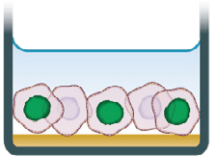
Dilute cytotoxicity reagent in medium at 1x and prepare cell treatments.

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® S3 Live-Cell Analysis System.

Day 0

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.

Day 1

2. Prepare cytotoxity 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. Seed cells and add prepared treatments

3.1. Seed your choice of cells (100 µL 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® S3 Live-Cell Analysis System and allow the plate to warm to 37°C for 30 minutes prior to scanning.

a. Objective: 4x (recommended 1 image per well or whole well) or 10x
b. Channel selection: Phase Contrast and Fluorescence
c. Scan type: Standard
d. Scan interval: Typically, every 2 hours, until your experiment is complete.

NOTE: A cytotoxic Index can be calculated on IncuCyte S3 Live-Cell Analysis System using the IncuCyte Cell-by-Cell Analysis Software Module (PN 9600 0031). This enables individual cell identification and subsequent classification into subpopulations based on properties including fluorescence intensity.

a. Scan type: Standard/Adherent Cell-by-Cell
b. Objective: 10x

For further details of this analysis module and it's application see:
www.essenbioscience.com/cell-by-cell

Related Products and Applications

A comprehensive range of fluorescent nuclear labeling and cell health reagents are available for use with the IncuCyte® S3 Live-Cell Analysis System to enable multiplexed measurements of apoptosis and proliferation alongside cytotoxicity.

Product	Cat No.	Amount
IncuCyte® NucLight Red BacMam 3.0 Reagent for nuclear labeling	4621	1 mL
IncuCyte® NucLight Green BacMam 3.0 Reagent for nuclear labeling	4622	1 mL
IncuCyte® NucLight Green Lentivirus Reagent (EF-1 α, Puro) for nuclear labeling	4624	0.2 mL
IncuCyte® NucLight Red Lentivirus Reagent (EF-1 α, Puro) for nuclear labeling	4625	0.2 mL
IncuCyte® NucLight Green Lentivirus Reagent (EF-1 α, Bleo) for nuclear labeling	4626	0.2 mL
IncuCyte® NucLight Red Lentivirus Reagent (EF-1 α, Bleo) for nuclear labeling	4627	0.2 mL
IncuCyte® NucLight Green Lentivirus Reagent (EF-1 α, Puro) for nuclear labeling	4475	0.6 mL
IncuCyte® NucLight Red Lentivirus Reagent (EF-1 α, Puro) for nuclear labeling	4476	0.6 mL
IncuCyte® NucLight Green Lentivirus Reagent (EF-1 α, Bleo) for nuclear labeling	4477	0.6 mL
IncuCyte® NucLight Red Lentivirus Reagent (EF-1 α, Bleo) for nuclear labeling	4478	0.6 mL
IncuCyte® Cytotox Red Reagent for counting dead cells	4632	5 µL x 5
IncuCyte® Cytotox Green Reagent for counting dead cells	4633	5 µL 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 µL
IncuCyte® Cell-by-Cell Analysis Software Module	9600-0031	1 Module

A complete suite of cell health applications is available to fit your experimental needs. Find more information at **essenbioscience.com**

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