IncuCyte® Apoptosis Assay

For the fluorescent detection of caspase-3/7 activation or phosphatidylserine externalization

This protocol provides an overview of the IncuCyte Apoptosis Assay methodology which uses mix-and read IncuCyte® Caspase 3/7 or Annexin V Reagents to detect apoptosis in real time. It is compatible with the IncuCyte® live-cell analysis system using your choice of cells and treatments. The highly flexible assay format can be combined with our range of IncuCyte® NucLight red nuclear labeling reagents or labeled cell lines for multiplexed measurements of proliferation and apoptosis in the same well.

Required materials

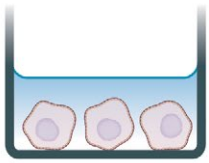
- IncuCyte® Caspase- 3/7 Green Apoptosis Reagent (Essen Bioscience Cat #4440)
  or
- IncuCyte® Caspase- 3/7 Red Apoptosis Reagent (Essen Bioscience Cat #4704)
  or
- IncuCyte® Annexin V Red Reagent (Essen BioScience Cat #4641)
  or
- IncuCyte® Anexin V Green Reagent (Essen BioScience Cat #4642)
- Poly-L-ornithine (Sigma P4957) – optional, for non-adherent cells
- Fibronectin (Sigma A7906) - optional, for non-adherent cells
- Flat bottom tissue culture plate (e.g., Corning 3595)

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 apoptosis in primary neuronal cultures, we recommend use of the IncuCyte Annexin V Red reagent to eliminate risk of green channel excitation issues in these sensitive cell lines.

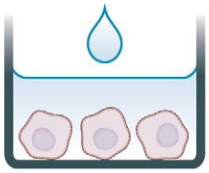
Adherent Cell Line Protocol

1 SEED CELLS



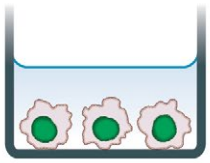
Seed cells (100 µL/well, 1,000 – 5,000) into a 96-well plate and incubate overnight.

2 PREPARE APOPTOSIS REAGENT AND TREAT CELLS



Prepare the desired treatments at 1x in medium containing IncuCyte Caspase-3/7 or Annexin V Reagents. 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**

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.

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 Apoptosis reagent preparation and cell treatment addition

2.1. Dilute apoptosis reagents in desired medium formulations
   a. If using Caspase-3/7 Reagents, dilute the reagent 1:1000 in complete medium (5 µM final concentration for Caspase-3/7 Green, and 0.5 µM for Caspase-3/7 Red).
   b. If using Annexin V reagents, solubilize Annexin V by adding 100 µL of complete medium or PBS. The reagents may then be diluted in complete medium containing at least 1 mM $CaCl_2$ for a final dilution of 1:200.

   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 apoptosis

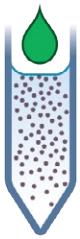
3.1. Place the cell plate into the IncuCyte Live-Cell Analysis System and allow the plate to warm to 37°C for 30 minutes prior to scanning.
   a. Objective: 10x or 20x
   b. Channel selection: Phase Contrast and Green or red (depending on apoptosis reagent used).
   c. Scan type: Standard (2-4 images per well).
   d. Scan interval: Typically, every 2 hours, until your experiment is complete.

Non-Adherent Cell Line Protocol

1 COAT PLATE

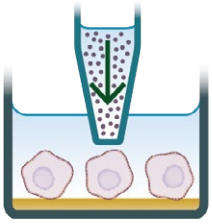
Coat plate with 0.01% poly-L-ornithine solution or 5 µL/mL fibronectin diluted in 0.1% BSA.

2 PREPARE INCUCYTE APOPTOSIS REAGENT AND TREATMENT



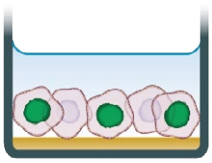
Dilute apoptosis reagent in medium and prepare cell treatments.

3 SEED CELLS AND ADD TREATMENT



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

4 LIVE CELL FLUORESCENT ANALYSIS



Capture images every 2-3 hours (20x or 10x) in the IncuCyte® System.

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 apoptosis reagent and treatments

2.1. Prior to cell seeding, dilute apoptosis reagents in desired medium formulation.
   a. If using Caspase-3/7 Reagents, dilute the reagent 1:1000 in complete medium (5 µM final concentration for Caspase-3/7 Green, and 0.5 µM for Caspase-3/7 Red).
   b. If using Annexin V reagents, solubilize Annexin V by adding 100 µL of complete medium or PBS. The reagents may then be diluted in complete medium containing at least 1 mM $CaCl_2$ for a final dilution of 1:200.

   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 200 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 containing caspase-3/7 or Annexin V 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 Caspase 3/7 or Annexin V. 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 apoptosis**

4.1. 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 Green or red (depending on apoptosis reagent used).

c. Scan type: Standard (2-4 images per well)

d. 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 cytotoxicity and proliferation alongside apoptosis.

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® Caspase-3/7 Red Reagent for apoptosis	4704	20 µL

A complete suite of cell health applications is available to fit your experimental needs. Find more information at
essenbioscience.com/cellhealth
essenbio.com/immuno-oncology

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