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® Annexin V Green Reagent (Essen Bioscience Cat #4642)
- Poly-L-ornithine (Sigma P4957)
  - optional, for non-adherent cells
- Fibronectin (Sigma F1141)
  - 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.

Day 0:
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.

2 PREPARE APOPTOSIS REAGENT AND TREAT CELLS

2.1. Dilute apoptosis reagents in desired medium formulations
   a. If using Caspase-3/7 Green Reagent, dilute the reagent 1:1000 in complete medium (5 μM final concentration).
   b. If using Caspase-3/7 Red Reagent, evaluate optimal reagent concentration by diluting the reagent 1:200 in complete medium, then make 2-fold dilutions (2.5, 1.25 and 0.5 μM final concentrations).
   c. 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 CaCl₂ 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 the 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
   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 CaCl2 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/well, 5,000 – 25,000 cells) into the coated 96-well plate. Immediately add apoptosis reagent ± treatments and triturate.

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.

<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>
<td>4621</td>
<td>1 mL</td>
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<td>IncuCyte® NucLight Green BacMam 3.0 Reagent for nuclear labeling</td>
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<td>IncuCyte® Cytotox Red Reagent for counting dead cells</td>
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<td>IncuCyte® Cytotox Green Reagent for counting dead cells</td>
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<td>IncuCyte® Annexin V Red Reagent for apoptosis</td>
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<td>IncuCyte® Annexin V Green Reagent for apoptosis</td>
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<td>IncuCyte® Caspase-3/7 Green Reagent for apoptosis</td>
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<tr>
<td>IncuCyte® Caspase-3/7 Red Reagent for apoptosis</td>
<td>4704</td>
<td>20 μL</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/cellhealth essenbio.com/immuno-oncology

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