PROTOCOL

IncuCyte® Apoptosis Assay with Annexin V Orange

For the fluorescent detection of phosphatidylserine externalization

This protocol provides an overview of the IncuCyte Apoptosis Assay methodology which uses mix-and-read IncuCyte® Annexin V Orange Reagent to detect apoptosis in real time. It is compatible with the IncuCyte® S3 Live-Cell Analysis System for Neuroscience (Cat. No. 4763) configured with the Orange/NIR Optical Module. It is not compatible with instruments configured with the Green/Red Optical Module, e.g. 4647. The highly flexible assay format can be combined with your choice of cells and treatments.

Required materials

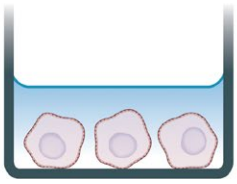
- IncuCyte® Annexin V Orange Reagent (Sartorius Cat# 4759)
- Poly-L-ornithine (Sigma Cat# P4957)
  – optional, for non-adherent cells
- Fibronectin (Sigma Cat# F1141)
  - optional, for non-adherent cells
- Flat bottom tissue culture plate (e.g., Corning Cat# 3595)

General guidelines

- 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 for Neuroscience, allow the plate to warm to 37 °C for 30 minutes prior to scanning

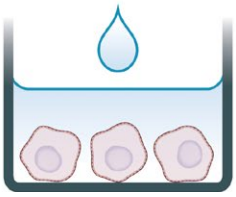
Adherent cell line protocol

1 SEED CELLS



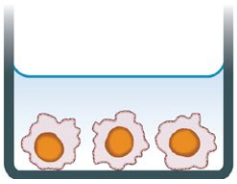
Seed cells (100 µL/well) into a 96-well plate.

2 PREPARE APOPTOSIS REAGENT AND TREAT CELLS



Prepare the desired treatments at 1x in medium containing IncuCyte Annexin V Reagent and add treatment.

3 LIVE CELL FLUORESCENT ANALYSIS



Capture images every 2-3 hours (20x or 10x) in the IncuCyte® S3 for Neuroscience. 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 S3 for Neuroscience 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. Solubilize Annexin V Orange Reagent 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 the appropriate wells of the 96-well plate.

3 **Live-Cell Imaging of apoptosis**

3.1. Place the cell plate into the IncuCyte S3 for Neuroscience 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 Orange.

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

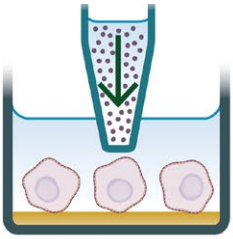
1 COAT PLATE

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

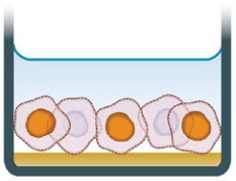
Dilute apoptosis reagent in medium and prepare cell treatments.

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® S3 for Neuroscience.

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 Cat# P4957) or 5 µg/mL fibronectin (Sigma Cat# 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, solubilize Annexin V Orange Reagent 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 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 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® S3 for Neuroscience 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 Orange.
c. Scan type: Standard (2-4 images per well).
d. Scan interval: Typically, every 2 hours, until your experiment is complete.

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

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