



Protocol

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® NuLight 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 (Sartorius Cat. No. 4440) *or*
- IncuCyte® Caspase-3/7 Red Apoptosis Reagent (Sartorius Cat. No. 4704) *or*
- IncuCyte® Annexin V Red Reagent (Sartorius Cat. No. 4641) *or*
- IncuCyte® Annexin V Green Reagent (Sartorius Cat. No. 4642) *or*
- IncuCyte® Annexin V Orange Reagent (Sartorius Cat. No. 4759) *or*
- IncuCyte® Annexin V NIR Reagent (Sartorius Cat. No. 4768)

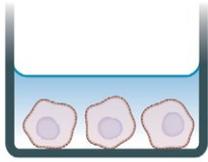
- Poly-L-ornithine (Sigma Cat. No. P4957) – optional, for non-adherent cells
- Fibronectin (Sigma Cat. No. F1141) – optional, for non-adherent cells
- Flat bottom tissue culture plate (e.g., Corning Cat. No. 3595)

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.

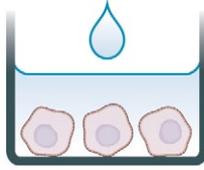
Adherent cell line protocol

1. Seed cells



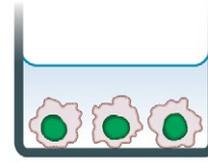
Seed cells (100 μ L/well, 1,000–5,000) into a 96-well plate and incubate overnight.

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 apoptosis reagent \pm treatment (100 μ L/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 μ L 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 Live-Cell Analysis 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. See table on page 4 for reagent specific recommendations.

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 μ L 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/Red/Orange/NIR (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.

NOTE: An apoptotic index can be calculated on IncuCyte S3 Live-Cell Analysis System using the IncuCyte Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031). This enables individual cell identification and subsequent classification into subpopulations based on properties including fluorescence intensity. To use this module, the following settings should be used:

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

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

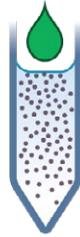
Non-adherent cell line protocol

1. Coat plate



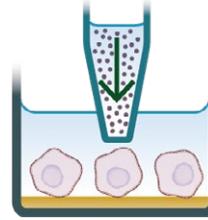
Coat plate with 0.01% poly-L-ornithine solution or 5 $\mu\text{L}/\text{mL}$ fibronectin diluted in 0.1% BSA.

2. Prepare IncuCyte apoptosis reagent and treatment



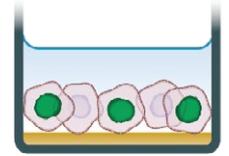
Dilute apoptosis reagent in medium and prepare cell treatments.

3. Seed cells and add treatment



Seed cells (100 $\mu\text{L}/\text{well}$, 5,000–25,000 cells) into the coated 96-well plate. Immediately add apoptosis reagent \pm 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 Cat. No. P4957) or 5 $\mu\text{g}/\text{mL}$ fibronectin (Sigma Cat. No. 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.

NOTE: See table on page 4 for reagent specific recommendations.

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.
- Objective: 10x or 20x.
 - Channel selection: Phase Contrast and Green/Red/Orange/NIR (depending on apoptosis reagent used).
 - Scan type: Standard (2–4 images per well).
 - Scan interval: Typically, every 2 hours, until your experiment is complete.

NOTE: An apoptotic index can be calculated on IncuCyte S3 Live-Cell Analysis System using the IncuCyte Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031). This enables individual cell identification and subsequent classification into subpopulations based on properties including fluorescence intensity. To use this module, the following settings should be used:

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

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

Cell health reagent recommendations and compatibility

Product	Cat. No.	Recommended concentrations
Reagents for IncuCyte Live-Cell Analysis System (Green/Red Optical Module)		
Annexin V Red	4642	Solubilize Annexin V by adding 100 μ L 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.
Annexin V Green	4641	Solubilize Annexin V by adding 100 μ L 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.
Caspase-3/7 Green	4440	Dilute Caspase-3/7 Green Reagent to 1:1000 in complete medium (5 μ M final concentration).
Caspase-3/7 Red	4704	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 μ M final concentrations).
Reagents for IncuCyte S3 Live-Cell Analysis System for Neuroscience (Orange/NIR Optical Module)		
Annexin V Orange	4759	Solubilize Annexin V by adding 100 μ L 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.
Annexin V NIR	4768	Solubilize Annexin V by adding 100 μ L 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. A spectral unmixing value of 2% is recommended. If additional optimization is needed, please refer to the Annexin V NIR Product Data Sheet for the optimization protocol.

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 Systems to enable multiplexed measurements of cytotoxicity and proliferation alongside apoptosis.

Product	Cat. No.	Amount
IncuCyte Live-Cell Analysis System (Green/Red Optical Module)		
IncuCyte® NuLight Green Lentivirus Reagent (EF-1 α , Puro) for nuclear labeling	4624	0.2 mL
IncuCyte® NuLight Red Lentivirus Reagent (EF-1 α , Puro) for nuclear labeling	4625	0.2 mL
IncuCyte® NuLight Green Lentivirus Reagent (EF-1 α , Bleo) for nuclear labeling	4626	0.2 mL
IncuCyte® NuLight Red Lentivirus Reagent (EF-1 α , Bleo) for nuclear labeling	4627	0.2 mL
IncuCyte® NuLight Green Lentivirus Reagent (EF-1 α , Puro) for nuclear labeling	4475	0.6 mL
IncuCyte® NuLight Red Lentivirus Reagent (EF-1 α , Puro) for nuclear labeling	4476	0.6 mL
IncuCyte® NuLight Green Lentivirus Reagent (EF-1 α , Bleo) for nuclear labeling	4477	0.6 mL
IncuCyte® NuLight Red Lentivirus Reagent (EF-1 α , Bleo) for nuclear labeling	4478	0.6 mL
IncuCyte® Cytotox Red Reagent for counting dead cells	4632	5 μ L x 5
IncuCyte® Cytotox Green Reagent for counting dead cells	4633	5 μ L 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 μ L
IncuCyte® Caspase-3/7 Red Reagent for apoptosis	4704	20 μ L
IncuCyte® Cell-By-Cell Analysis Software Module	9600-0031	1 module
IncuCyte Live-Cell Analysis System for Neuroscience (Orange/NIR Optical Module)		
IncuCyte® Annexin V Orange Reagent for apoptosis	4759	100 tests
IncuCyte® Annexin V NIR Reagent for apoptosis	4768	100 tests
IncuCyte® NuLight Orange Lentivirus Reagent (EF-1 α , Puro) for nuclear labeling	4771	0.2 mL
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 www.sartorius.com/incucyte

For additional product or technical information, please e-mail us at AskAScientist@sartorius.com or visit our website at www.sartorius.com/incucyte

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