

IncuCyte® Immune Cell Killing Assay

For measurements of tumor cell death

This protocol provides an overview for the measurement of immune cell killing of adherent or non-adherent target tumor cells. The flexible assay format is suitable for cytotoxic T cell killing and antibody-dependent cell-mediated cytotoxicity (ADCC) assays using a co-culture methodology that combines direct measurements of tumor cell death with no-wash, mix-and-read protocols. This method utilizes either our IncuCyte® Caspase 3/7 reagent, a substrate that is cleaved during target cell apoptosis to release a green-fluorescent DNA dye that stains the nuclear DNA, or IncuCyte® Annexin V reagent which labels externalized phosphatidylserine (PS) moieties. IncuCyte® image analysis software enables automated detection and selective quantitation of tumor cell death in real time.

Required materials

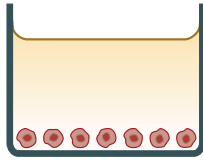
- IncuCyte® Caspase- 3/7 Apoptosis Reagent (Essen Bioscience Cat #4440) or
- IncuCyte® Annexin V Green Reagent (Essen BioScience Cat #4642) or
- IncuCyte® Annexin V Red Reagent (Essen BioScience Cat #4641)
- Poly-L-ornithine (Sigma P4957), for non-adherent cells or
- Fibronectin (Sigma A7906), for non-adherent cells
- Flat bottom tissue culture plate (e.g., Corning 3595)
- Target cells of interest (non-adherent target cells are required to be labeled with NuLight live-cell labeling reagent to enable tumor cell counting).
- IncuCyte® NuLight Red Lentivirus Reagent (Essen BioScience Cat # 4476)
- IncuCyte® NuLight Green Lentivirus Reagent (Essen BioScience Cat # 4475)
- Immune (effector) cells of interest
- 96-well microplate (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

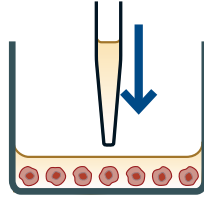
Immune cell killing of adherent tumor cells protocol

1 SEED TARGET CELLS



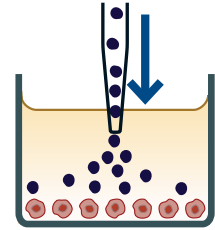
Seed tumor cells (100 μ L/well, 1,000 to 3,000/well) into the 96-well plate.
Optional: Target cells can be labeled with IncuCyte® NucLight Red live-cell labeling reagent (Essen BioScience 4476) to enable simultaneous tumor cell counting.

2 TREAT CELLS



Aspirate the medium and add the Caspase 3/7 reagent or Annexin V reagent (50 μ L/well) and desired treatments (50 μ L/well) at 4x final assay concentrations.

3 ADD IMMUNE CELLS



Add your choice of immune cells (100 μ L/well, 10,000 to 30,000/well) to a 96-well plate.

Day 0

1 Seed target cells

- 1.1. Seed target cancer cells (100 μ L per well) at an appropriate density into a 96-well flat-bottom plate such that by day 1 the cell confluency is approximately 20%. The seeding density will need to be optimized for each tumor cell line used; however, we have found that 1,000 to 3,000 cells per well are reasonable starting points.
 - a. Target cell growth can be monitored using the IncuCyte® live-cell analysis system and confluence algorithm.
 - b. **Optional:** Target cells can be labeled with NucLight Red live-cell labeling reagent (Catalog # 4475 or 4476) to enable simultaneous real-time counting of viable tumor cells.

- 2.2. Remove the cell plate from the incubator and aspirate off growth medium.
- 2.3. Add 50 μ L each of the prepared apoptosis reagent and treatments from step 2.1 above.
NOTE: For treatment controls, add 50 μ L of assay medium.

3 Add immune cells

- 3.1. Count chosen effector cells (e.g. T cells, PBMCs) and prepare a cell suspension at a density of 100,000 to 300,000 cells/mL (100 μ L per well, 10,000 to 30,000 cells/well). It is recommended that different target-to-effector cell ratios are tested (e.g., 1:5, 1:10).
NOTE: Assay duration may be reduced by pre-activating the effector cells before addition to assay plate, however, this may require a higher initial seeding density of target cells.
- 3.2. Seed 100 μ L of effector cells into the appropriate wells of the cell plate to achieve a final assay volume of 200 μ L. Allow plates to settle on level surface at ambient temperature for 30 minutes.
- 3.3. Place the assay plate into the IncuCyte live-cell analysis system and schedule 24 hour repeat scanning:
 - a. Objective: 10x
 - b. Channel selection: Phase Contrast + “Green” or “Red” depending on apoptosis reagent and target cell label used
 - c. Scan type: Standard (2 images per well)
 - d. Scan interval: Every 3 hours

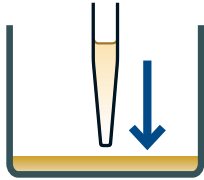
Day 1

2 Prepare apoptosis reagent and treatments

- 2.1. Dilute apoptosis reagents, ensuring compatibility of target cell label and apoptotic marker, and treatments (e.g., T cell stimuli, antibodies, cytokines) at 4x final assay concentration in desired assay medium.
 - a. If using caspase-3/7, dilute reagent to a concentration of 20 μ M (1:250 dilution), sufficient for 50 μ L per well.
 - b. If using Annexin V reagents, 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 CaCl₂ for a dilution of 1:50, sufficient for 50 μ L per well.

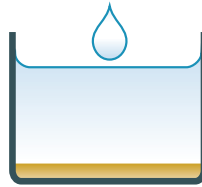
Immune cell killing of non-adherent tumor cells protocol

1 COAT PLATE



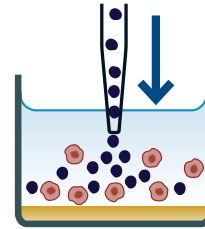
Coat plate surface to ensure even target cell coverage e.g. Poly-L-ornithine solution.

2 PREPARE TREATMENTS



Prepare Annexin V reagent (50 μ L/well) and desired treatments (50 μ L/well) at 4x final assay concentrations.

3 ADDITION OF TARGET AND EFFECTOR CELLS



Add NuLight labeled target cells (50 μ L/well, 10,000 to 20,000/well) and immune cells (50 μ L/well, 100,000 to 200,000/well) to a 96-well plate.

Day 1

1 Coat plate

1.1. Coat a 96-well flat bottom plate with relevant coating matrix. We recommend coating with 50 μ L of either 0.01% poly-L-ornithine solution or 5 μ g/mL fibronectin 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. Choice of coating will need to be determined prior to the assay for target cells of interest.

2 Reagent and treatment preparation

2.1. Prepare the following reagents in medium:

- Test materials (e.g. T cell stimuli, antibodies, cytokines; 50 μ L per well, prepared at 4x final assay concentration).
- Apoptosis detection reagent (ensure compatibility of cell label and apoptotic marker), IncuCyte® Annexin V Reagent (Cat # 4641 or 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 CaCl₂ for a dilution of 1:50 (4x final assay concentration, 50 μ L per well).

NOTE: Although either the IncuCyte® Annexin V or Caspase-3/7 reagents can be used to detect immune cell killing of target cells we recommend that the Annexin V reagent is used for non-adherent target cells. Non-adherent target and effector cell types can have very similar nuclear sizes negating the use of size filters to remove Caspase-3/7 labeled effector nuclei from the analysis. Additionally, we have observed raised levels of caspase 3/7 activity in some non-adherent cell types, particularly at higher confluency, which can interfere with the interpretation of immune cell driven target cell death. In our experience the Annexin V reagent labels fewer effector cells and provides lower non-specific background.

2.2. Add all prepared reagents to assay plate to achieve 100 μ L per well.

3 Add immune cells

3.1 Count labeled target cells and prepare a cell suspension at a density of 40,000 – 80,000 cells/mL (seed 50 μ L per well, 10,000 to 20,000 cells/well). Target cells can be labeled with NuLight Red or Green live-cell labeling reagent (Cat # 4476 or 4475) to enable simultaneous real-time counting of viable tumor cells.

3.2. Count chosen effector cells (e.g. T cells, PBMCs) and prepare a cell suspension at a density of 400,000 to 800,000 cells/mL (50 μ L per well, 100,000 to 200,000 cells/well). It is recommended that different target-to-effector cell ratios are tested (e.g. 1:5, 1:10).

NOTE: Assay duration may be reduced by pre-activating the effector cells before addition to assay plate, however, this may require a higher initial seeding density of target cells.

3.3. Add target and effector cells to assay plate to achieve a final assay volume of 200 μ L. Allow plates to settle on level surface at ambient temperature for 30 minutes.

3.4. Place the assay plate into the IncuCyte® instrument and schedule 24 hour repeat scanning:

- Objective: 4x
- Channel selection: Phase Contrast + “Green” and “Red”
- Scan type: Standard (2 images per well)
- Scan interval: Every 2–3 hours

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 apoptosis and proliferation alongside cytotoxicity.

Product	Cat No.	Amount
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

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

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