PROTOCOL

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 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

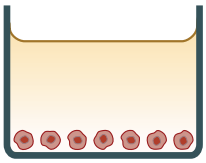
- IncuCyte® Caspase- 3/7 Apoptosis Reagent (Essen Bioscience Cat #4440) or
- IncuCyte® Annexin V Green Reagent (Essen BioScience Cat #4642) or
- IncuCyte® Annexin V Red Reagent (Essen BioScience Cat #4641)
- Poly-L-ornithine (Sigma P4957), *or* Fibronectin (Sigma A7906), for non-adherent cells
- Effector cell culture media
- Target cells of interest (non-adherent target cells are required to be labeled with NucLight live-cell labeling reagent to enable tumor cell counting).
- IncuCyte® NucLight Red Lentivirus Reagent (Essen BioScience Cat # 4476)
- IncuCyte® NucLight 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 nonadherent 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

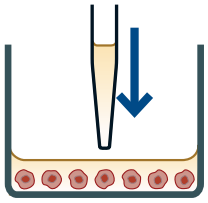
Immune cell killing of adherent tumor cells protocol

1 SEED TARGET CELLS



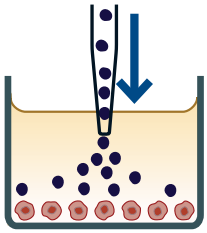
Seed tumor cells (100 µL/well, 1,000 to 3,000/well) into the 96-well plate. Optional: Target cells can be labeled with IncuCyte® NucLightRedlive-cell labeling reagent (EssenBioScience 4476) to enable simultaneous tumor cell counting.

2 TREAT TARGET CELLS



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

3 ADD IMMUNE CELLS



Add your choice of immune cells (100 µL/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 µL 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.

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 green, dilute reagent to a concentration of 20 µM (1:250 dilution), sufficient for 50 µL per well.

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 dilution of 1:50, sufficient for 50 µL per well.

2.2 Remove the cell plate from the incubator and aspirate off growth medium.

2.3 Add 50 µL each of the prepared apoptosis reagent and treatments from step 2.1 above.

NOTE: For treatment controls, add 50 µL of assay medium.

Add immune cells

3 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 µL 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 µL of effector cells into the appropriate wells of the cell plate to achieve a final assay volume of 200 µL. 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

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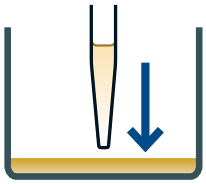
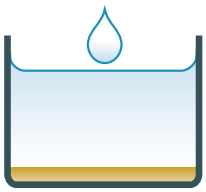
Immune cell killing of non-adherent tumor cells protocol

1 COAT PLATE



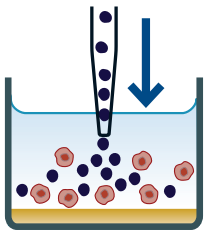
Coat plate surface to ensure even target cell coverage e.g. Poly-L-ornithine

2 PREPARE TREATMENTS



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

3 ADDITION OF TARGET AND EFFECTOR CELLS



Add NucLight labeled target cells (50 µL/well, 10,000 to 20,000/well) and immune cells (50 µL/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 µL of either 0.01% poly-L-ornithine solution or 5 µg/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:

a. Test materials (e.g. T cell stimuli, antibodies, cytokines; 50 µL per well, prepared at 4x final assay concentration).

b. 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 µL 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 µL 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. Nonadherent 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 µL 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 µL per well, 10,000 to 20,000 cells/well). Target cells can be labeled with NucLight 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 µL per well, 100,000 to 200,000 cells/well). It is recommended that different target-toeffector cell ratios are tested (e.g. 1:5, 1:10).

NOTE: Assay duration may be reduced by preactivating 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 µL. 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:

a. Objective: 4x

b. Channel selection: Phase Contrast + "Green" and "Red"

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

d. 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® 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 testsL
IncuCyte®Caspase-3/7 Green Reagent for apoptosis	4440	20 µL

A complete suite of cell health applications is available to fit yourexperimental 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** visit our website at **essenbioscience.com** or call
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