



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-3/7 Reagent, a substrate that is cleaved during target cell apoptosis to release a red or green fluorescent DNA dye that stains the nuclear DNA, or IncuCyte® Annexin V Apoptosis 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 Green Apoptosis Reagent (Sartorius Cat. No. 4440) or IncuCyte® Caspase-3/7 Red Apoptosis Reagent (Sartorius Cat. No. 4704) or IncuCyte® Annexin V Green Reagent (Sartorius Cat. No. 4642) or IncuCyte® Annexin V Red Reagent (Sartorius Cat. No. 4641)
- Poly-L-ornithine (Sigma Cat. No. P4957), or Fibronectin (Sigma Cat. No. A7906), for non-adherent cells
- Effector cell culture media
- Target cells of interest (non-adherent target cells are required to be labeled to enable tumor cell quantification)
- IncuCyte® NuLight Red Lentivirus Reagent (Sartorius Cat. No. 4476)
- IncuCyte® NuLight Green Lentivirus Reagent (Sartorius Cat. No. 4475)
- IncuCyte® CytoLight Rapid Green Reagent (Sartorius Cat. No. 4705)
- IncuCyte® CytoLight Rapid Red Reagent (Sartorius Cat. No. 4706)
- Immune (effector) cells of interest
- 96-well microplate (e.g., Corning® 3595)

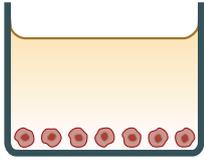
General guidelines

- 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.

IncuCyte® Immune Cell Killing Assay Protocol

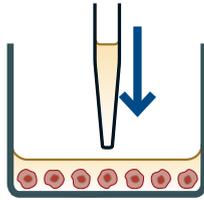
Immune cell killing of adherent tumor cells protocol

1. Seed target cells



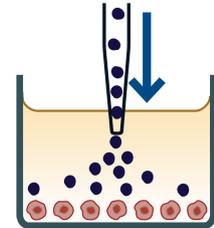
Seed tumor cells (100 μ L/well, 1,000 to 3,000/well) into the 96-well plate. Optional: Target cells can be labeled with IncuCyte® live-cell labeling reagent to enable simultaneous tumor cell quantification.

2. Treat target 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



95% media replacement. Plate rAstrocytes. Begin IncuCyte® S3 scanning.

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 NuLight Lentiviral Reagent (Sartorius Cat. No. 4475 or 4476) or Cytolight Rapid Reagents (Sartorius Cat. No. 4705 or 4706) to enable simultaneous real-time quantification 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 μ M (1:250 dilution), sufficient for 50 μ L per well.
 - b. If using Caspase-3/7 Red Reagent, evaluate optimal reagent concentration by diluting the reagent 1:50 in complete medium, then make 2-fold dilutions (10, 5 and 2.5 μ M final concentrations), sufficient for 50 μ L per well.
 - c. 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 CaCl_2 for a dilution of 1:50, sufficient for 50 μ L per well.

- 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.

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

3.

Add immune cells

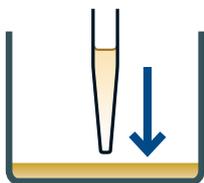
- 3.1 Count chosen effector cells (e.g. T cells, PBMCs) and prepare a cell suspension at a density of 100,000 to 300,000 cells/mL (100 μ L per well, 10,000 to 30,000 cells/well). It is recommended that different target-to-effector cell ratios are tested (e.g., 1:5, 1:10).

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 or 20X
 - 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)
NOTE: If the IncuCyte Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031) is activated, use non-adherent Cell-by-Cell image acquisition, 4 images/well.
 - d. Scan interval: Every 3 hours

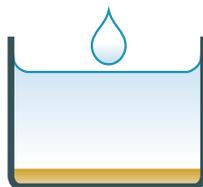
Immune cell killing of non-adherent tumor cells protocol

1. Coat plate



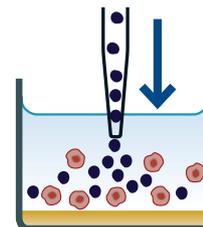
Coat plate surface to ensure even target cell coverage e.g. Poly-L-ornithine

2. Prepare treatments



Prepare Annexin V Reagent (50 $\mu\text{L}/\text{well}$) and desired treatments (50 $\mu\text{L}/\text{well}$) at 4X final assay concentrations.

3. Addition of target and effector cells



Add NuLight labeled target cells (50 $\mu\text{L}/\text{well}$, 10,000 to 20,000/well) and immune cells (50 $\mu\text{L}/\text{well}$, 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 $\mu\text{g}/\text{mL}$ 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 μL per well, prepared at 4X final assay concentration).
- Apoptosis detection reagent (ensure compatibility of cell label and apoptotic marker), IncuCyte® Annexin V Reagent (Sartorius Cat. No. 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 CaCl_2 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. 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 50 μL each of the prepared apoptosis reagent and treatments from Step 2.1 for a total volume of 100 μL .

3.

Add immune cells

- 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 NuLight Red or Green Lentivirus Reagent (Sartorius Cat. No. 4476 or 4475) to enable simultaneous real-time counting of viable tumor cells. Target cells can also be labeled with CytoLight Rapid Green or Red Reagent (Sartorius Cat. 4705 or 4706) for real-time quantification of tumor cell viability.
- 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-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.

- 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.
- Place the assay plate into the IncuCyte® instrument and schedule 24 hour repeat scanning:
 - Objective: 4X or 20X with Cell-by-Cell
 - Channel selection: Phase Contrast + "Green" and "Red"
 - Scan type: Standard (2 images per well)
NOTE: If the IncuCyte Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031) is activated, use non-adherent Cell-by-Cell image acquisition, 4 images/well.
 - Scan interval: Every 2-3 hours

Analysis Guidelines

Analysis with IncuCyte Basic Analyzer

When defining your analysis definition, select "Basic Analyzer" from the list of options.

- Select appropriate images to describe the biology in your plate across time, including example control wells.
- Define a fluorescence mask for target cell expression to quantify this population.
- Define a fluorescence mask for the cell health indicator in target cells, if included.
 - Use area and eccentricity filters to avoid masking any labeled effector cells.

NOTE: The phase confluence mask is of limited use as this will mask all target and effector cells in the well.

- When looking at results, use target cell fluorescence to quantify target cell proliferation.
 - If using a nuclear marker with adherent target cells, use Count of Objects.
 - If using a cytoplasmic marker with adherent target cells, use Total Fluorescence Area or Fluorescence Confluence.
 - For non-adherent target cells, use Total Fluorescence Area or Fluorescence Confluence regardless of cell labeling.
- Fluorescence of the cell health indicator will indicate apoptosis in the target cell population which can be normalized for target cell number. Again, use metrics of cell Total Fluorescence Area or Fluorescence Confluence.

Analysis with IncuCyte Cell-by-Cell Analysis Module

Adherent target cells

Use above recommendations with Basic Analyzer for tracking the target cell population, but use Non-adherent Cell-by-Cell Analysis to track properties of the effector cell population.

NOTE: It is possible to use Adherent Cell-by-Cell Analysis to mask target cells, but it is very difficult to not include effector cells in the mask and is very dependent on the contrast level of the target cells.

For effector cell masking when defining your analysis definition, select "Non-adherent Cell-by-Cell" from the list of options (only visible if images acquired with 20X and if license is active):

- Select appropriate images to describe biology in your plate across time, including example control wells. Include effector only wells if present in the plate.
- Define a phase mask to describe individual effector cells using control sliders (see Cell-By-Cell Analysis Guideline document for full details of software.)
- When looking at total population metrics, use object count to define proliferation of cells.
- If interested in the health of effector cells, set up a classification job on the segmented cells, based on fluorescence intensity, to define live and apoptotic effector cells.
 - Use percentage of apoptotic cells as indicator of cell health of effector cells.

Non-adherent target cells

When defining your analysis definition, select "Non-Adherent Cell-by-Cell" from the list of options (only visible if images acquired with 20X and if license is active):

- Select appropriate images to describe biology in your plate across time, including example control wells.
- Define a phase mask for all true cell objects (target and effector) using control sliders (see Cell-By-Cell Analysis Guideline document for full details of software.)
 - For target cells in the image, it may not be possible to mask as individual cells due to clumping—this will not cause an issue for overall quantification of target cell biology.
- Once the segmentation job has run, set up a two metric classification job based on red and green fluorescence intensity to identify sub populations:
 - Color 1 (e.g. NuLight Red)—Target cells (fluorescent cells) and effector cells (non-fluorescent).
 - Color 2 (e.g. Annexin V Green)—Live and apoptotic cells.

To view results we recommend the following metrics:

- Target cell properties—These will be from the high Color 1 intensity population.
 - For proliferation, create a metric (+) of high Color 1 (the population you are interested in) channel Color 1 integrated intensity per area (image or mm²), or channel phase area per image.
 - This will provide a total fluorescence area or phase area in the well for the target cell population. As it is difficult to mask individual target cells, avoid the count metric.
 - If using CytoLight Rapid dyes, use the phase area as the fluorescence intensity may drop over time.
 - For target cell death, create a metric (+) of high Color 1 and 2 phase count normalized by high Color 1 to provide an apoptotic index for the target cell population.
- Effector cell properties—These will be from low Color 1 intensity population.
 - For proliferation, again create a metric (+) of low Color 1, phase object count. Effector cells can normally be masked individually, so a readout of count can be used.
 - For effector cell death, create a metric (+) of low Color 1 and high Color 2 phase count normalized by low Color 1.

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® 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® CytoLight Rapid Green Reagent	4705	100–1000 tests
IncuCyte® CytoLight Rapid Red Reagent	4706	100–1000 tests
IncuCyte® Cell-by-Cell Analysis Software Module	9600-0031	1 module

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