Protocol

IncuCyte®– iQue3 Workflow for the Immune Cell Killing Assay

This protocol provides a detailed workflow for the combination of the IncuCyte and iQue3 to facilitate measurement of immune cell mediated killing of adherent or non-adherent target tumor cells and T-cell activation (TCA). This assay workflow describes how the two platforms can be used both simultaneously and concurrently to provide a deeper understanding of immune cell killing (ICK). The workflow allows for (1) direct measurements of tumor cell death with a no-wash, mix-and-read protocol using the IncuCyte ICK application and co-culture methodology; (2) quantification of morphological and spatial data using the IncuCyte Cell-by-Cell analysis software and (3) quantification of T-cell activation markers and cytokine release using the Intellicyt Human T Cell Activation Cell and Cytokine Profiling Kit (TCA kit). The ICK assay can be directly transferred between the two platforms with minimal sample manipulation.

Required materials

- Target cells of interest: cells are required to be stably expressing the NucLight Green Lentivirus (Essen BioScience Cat. No. 4475)
- Poly-L-ornithine (Sigma Cat. No. P4957) for coating plate when using non-adherent target cells
- IncuCyte Annexin V Red Reagent (Essen BioScience Cat. No. 4641)
- Immune (effector) cells of interest
- Effector cell culture media
- Effector cell activator (e.g. CD3/CD28 Dynabeads®)
- Human T Cell Activation Cell and Cytokine Profiling Kit-TCA kit (Intellicyt Cat. No. 90560)
- QBeads® if temporal cytokine analysis desired (Intellicyt Assay Builder: IFNγ option 1 and TNFα option 2).
- 96-well flat-bottom microplate (e.g. Corning Cat. No. 3595)
- 96-well v-bottom microplate (e.g. Costar Cat. No. 3363)
- Accutase (e.g. Gibco Cat. No. A1110501)

Suggested materials

- Plate washer: a number of points in the protocol require a wash step and while plates can be 'flicked' in order to remove unwanted supernatant, a plate washer is highly recommended.

  NOTE: There is no recommended plate washer and instrument settings must be optimized before use.

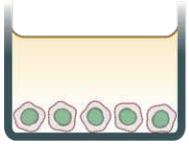
General guidelines

- 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.
- It is strongly recommended that the following controls are included on the plate: (1) a non-activated PBMC control in co-culture with target cells (2) target cell in mono-culture (3) activated PBMC in mono-culture.
- 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.
- For the final cytokine sample the 10 µl volume must be taken prior to cell transfer and assayed in a separate plate to the antibody labeled cells.
- Cytokine standards require fresh cell media to ensure the reproducibility and reliability of your data.
- The optional Cell Proliferation and Encoder Dye (B/Green) from TCA kit cannot be included in this experiment due to the use of NucGreen target cells.

Immune cell killing of adherent or non-adherent tumor cells protocol

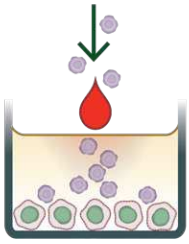
IncuCyte | iQue3

1. Seed target cells (Day 0)



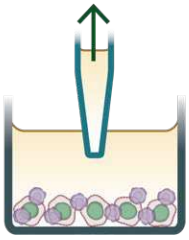
Seed NucLight Green labeled tumor cells (100 µL/well) into 96-well plate.

2. Add activators and PBMCs (Day 0)



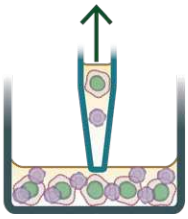
Add Annexin V Red reagent (25 µL/well), desired treatments (25 µL/well) and your choice of immune cells (50 µL/well).

3. Take cytokine samples (daily)



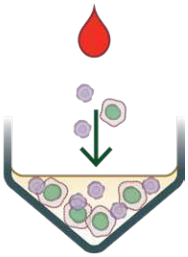
10 µL supernatant samples may be taken daily to allow temporal cytokine analysis.

4. Lift cells for analysis (endpoint)



Spin and wash plate with PBS (100 µL/well). Gently remove and lift cells using 50 µL Accutase for 10 minutes.

5. Label using TCA kit (endpoint)



Transfer to V-bottom plate and label using antibody detection panel (10 µL/well) from TCA kit.

1. Seed target cells

1.1 **Coat plate for non-adherent cells**: Coat a 96-well flat bottom plate with relevant coating matrix. It is recommended that wells are coated using 50 µL of 0.01% poly-L-ornithine solution. Coat plates for 1 hour at RT, remove solution from wells, allow plates to dry for 30 minutes prior to cell addition.

1.2 **Seed target cancer cells**: An appropriate density of cells must be seeded (100 µL/well). 2,000 to 5,000 cells/well for adherent cells (seeded in the morning) and 10,000 to 20,000 cells/well for non-adherent (seeded prior to assay) are reasonable starting points.

NOTE: NucLight Green expressing cells must be used to allow integration of IncuCyte and iQue3. NucLight Red cells are not compatible with the Human T Cell Activation Cell and Cytokine Profiling Kit (TCA kit).

2. Reagent and treatment preparation

2.1 Prepare the following reagents in medium:

a. Test materials (e.g. T-cell stimulants, antibodies, cytokines; 25 µL/well, prepared 8x FAC).

b. Apoptosis detection reagent, IncuCyte Annexin V Red (Cat. No. 4641): solubilize Annexin V by adding 100 µL of complete medium or PBS. The reagent can then be diluted as required in complete medium containing at least 1 mM $CaCl_2$ (25 µL/well at 8x FAC, for a final dilution of 1:200).

2.2 Add all reagents to assay plate to achieve a volume of 150 µL/well.

3. Add immune cells

3.1 Prepare an effector cell suspension at an appropriate density. It is recommended that different target-to-effector ratios are tested (e.g., 1:3, 1:5).

NOTE: Cell Proliferation and Encoder Dye (B/Green) from the TCA kit cannot be included in this assay due to the presence of NucGreen Target cells.

3.2 Seed 50 µL/well to achieve a total assay volume of 200 µL. Allow plates to settle on level surface at RT for 30 minutes.

3.3 Place the assay plate into the Incucyte Live-Cell Analysis System, allow to warm to 37° C for 30 minutes. Schedule 24 hour repeat scanning:

a. Scan Type: Non-adherent Cell-by-Cell or Standard (4-images per well)

b. Objective: 20x

c. Channel selection: Phase + Green + Red (if including apoptosis reagent)

d. Scan interval: 3 hours

4. Optional: Daily cytokine samples

4.1 Combine lyophilized standards from the QBeads kit and add 200 µL fresh culture media incubate at RT for 15 minutes without trituration.

4.2 Prepare an 11-point 1:3 serial dilution of solubilised standards. Plate 10 µL of each dilution into duplicate wells of a V-bottom plate, with concentration going from low to high (Rows A and B, 2-12). Place 10 µL of media only in 1st wells as a control.

4.3 10 µL of supernatant from each sample on the assay plate should be transferred to the remaining empty wells of the V-bottom plate, i.e. Rows C-H.
NOTE: Ensure removal and replacement of plate from IncuCyte will not interrupt scheduled 3 hour scans.

4.4 Capture beads are diluted by 18 times volume with fresh media. Add 190 µL/well and incubate at RT in the dark for 1 hour.

4.5 Centrifuge at 300 x g 5 minutes and aspirate supernatant. Re-suspend beads in residual liquid by strong shaking (3000 rpm 1 minute).

4.6 Add 10 µL/well Cytokine Detection, followed by a quick spin (300 x g 5 seconds) and brief shake (2000 rpm 20 seconds). Leave in the dark at RT for 2 hours.

4.7 Add 100 µL/well wash buffer, spin at 300 x g 5 minutes and aspirate.

4.8 Re-suspend beads in residual liquid and add 20 µL/well wash buffer.

4.9 Run plate on iQue3 with TCA template. Optimization of protocol may be necessary to achieve required results. A minimum sip time of 4 seconds is recommended.

5. Endpoint T-cell subset and cytokine analysis

5.1 For endpoint cytokine analysis, remove 10 µL of supernatant from each sample well and follow section 4.

5.2 Centrifuge plate at 300 x g for 5 minutes and remove supernatant gently using multichannel pipette, leaving at least 50 µL media in well.

5.3 Wash once with PBS and centrifuge at 300 x g for 5 minutes to ensure all cells remain in the wells.

5.4 Gently remove PBS and add 50 µL/well Accutase for 10 minutes at 37° C followed by a 2 minute shake at 1400 rpm.

5.5 Add 100 µL media to quench Accutase, triturate sample and then transfer to a V-bottom plate.

NOTE: Use a microscope to check that cells have been lifted and transferred from the cell culture plate, if cells still remain repeat steps 5.3-5.4.

5.6 Centrifuge plate at 300 x g for 5 minutes, aspirate supernatant and re-suspend in 10 µL of fresh media.

5.7 Dilute Cell Viability Dye 1:500 into Antibody Detection Panel. Add 10 µL/well followed by quick spin (300 x g 5 seconds), brief shake (2000 rpm 20 seconds). Leave at RT in the dark 1 hour.

5.8 Add 100 µL wash buffer and centrifuge at 300 x g for 5 minutes.

5.9 Re-suspend cells in residual liquid and add 20 µL/well wash buffer.

5.10 Run plate on iQue3 with TCA kit template. Optimization may be necessary to achieve required results. A minimum sip time of 4 seconds is recommended.

6. Analysis using ForeCyt

6.1 TCA kit comes with a templated analysis. This includes automated compensation that should not require changes for this assay.

6.2 A change in the gating should be included in order to exclude your target cells from the gates:

a. All events: select cell populations using SSC-H vs FSC-H.
b. Single cells: identify single cells using FSC-H vs FSC-A.
c. NucGreen exclusion: BL1-H vs FSC-H will identify two populations. Low green are effector cells and high green target cells.
d. Live/Dead cells: RL1-H vs SSC-H can then be used to exclude any dead cells.
e. Continuation of TCA kit gates: copy gates onto new population to allow exclusion of target cells.

A complete suite of cell health and T-cell biology applications is available to fit your experimental needs. Find more information at **www.sartorius.com/incucyte** and **www.sartorius.com/intellicyt**

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

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