SARTORIUS

Product Protocol

T Cell Killing in Single Spheroids

Background

This protocol describes a simple workflow for dissociating 3D spheroids and transferring them onto the iQue® for analysis. This protocol is applicable to T cell killing assays, compatible with iQue® Human T Cell Activation, iQue® Human T Cell Killing and iQue® Human T Cell Exhaustion Kits. The dissociation protocol has been optimized to minimize sample manipulation during cell transfer. This method can also be used in a combined workflow with temporal analysis of killing by Incucyte® live cell imaging platform followed by cytokine and cell subset measurements using the iQue® advanced flow cytometer.

Required Materials

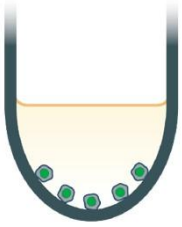
Target cells of interest (adherent): stably expressing Incucyte® Nuclight Green Lentivirus Reagent (Sartorius: 4475)
Matrigel® (Corning Cat. No. 356234)
Effector cell culture media
Effector cells of interest
Effector cell activator (e.g. Dynabeads CD3/CD28)
96-well ULA microplate (e.g. S-BIO PrimeSurface)
96-well V-bottom microplate (e.g. Costar Cat. No. 3363)
Cell Recovery Solution (e.g. Corning Cat. No. 354253)
Accutase (e.g. Gibco Cat.No. A1110501)
PBS
PBS + 2% FBS

General Guidelines

- This dissociation protocol can be applied to T cell killing assays. Follow the guide to set up a T cell killing assay and analyze with the T cell characterization kit of your choice.
- Optimization of target cell density is required to ensure spheroids form with the desired size (e.g. 200–500 µm). Seeding density will need to be optimized for each cell line used, however we recommend a range of 1,000 to 5,000 cells per well.
- Some cell lines may require the addition of a basement membrane extract, typically 1.5 – 2.5% Matrigel®, to promote tight spheroid formation. Optimization is required depending on the target cell used.
- Due to the spheroid dissociation steps, this method requires cytokine samples to be removed from the assay plate and analyzed separately to the cell subsets. The standard protocol for the iQue® T cell characterization kits describes how to analyze these parameters in multiplex and therefore must be modified to separate the antibody labelling and Qbead® cytokine detection steps.

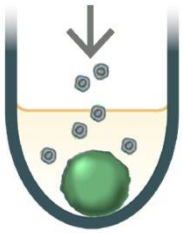
Quick Guide

1. Seed Target Cells
Day 0



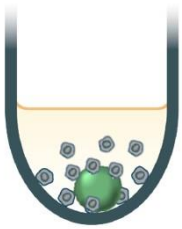
Seed target cells at appropriate density. Add Matrigel® if required. Centrifuge 125 xg 10 mins.

2. Add Effector Cells
Day 3



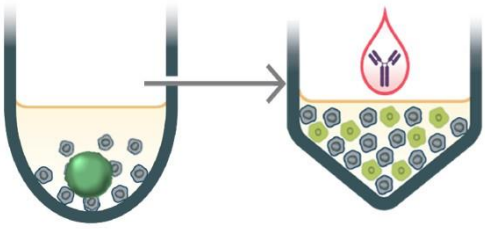
After 72 h, add effector cells (50 µL/well) and activators of interest (50 µL/well)

3. Monitor killing of Spheroids.
Day 3 - 11



Optional: Monitor immune cell killing of spheroids over time (e.g. using Incucyte®). Take cytokine samples and store at -20 °C until analysis.

4. Select End Point, Dissociate Spheroids & Label.
(Day 3 – 11)



Select appropriate end point (once killing has occurred). Dissociate spheroid and label with antibodies. Assess T cell phenotypes and target cell count on the iQue® .

T Cell Killing Protocol

Day 0

1. Seed target cells

1.1. Matrigel not required

1.1.1. Seed target cells into a 96- well ULA plate at an appropriate density (100 µL/well).

1.1.2. Centrifuge the ULA plate (125 xg, 10 minutes) at RT.

1.2. Matrigel required

1.2.1. Seed target cells into a 96- well ULA plate at an appropriate density (75 µL/well).

1.2.2. Centrifuge the ULA plate (125 xg, 10 minutes) at RT.

1.2.3. Add Matrigel (25 µL/well) to give a final assay concentration of 1.5-2.5% v/v.

NOTE: Depending on the target cell line used, Matrigel concentration may need to be optimized to ensure compact spheroid formation.

2. Spheroid formation

To monitor spheroid formation, place the cell plate into the Incucyte® Live-Cell Analysis System and schedule 24 hour repeat scans:

a. Scan Type: Spheroid
b. Channel Selection: Single spheroid, Phase + Brightfield and Green.
c. Objective: 4x
d. Scan Interval: 6 h

NOTE: Using an Incucyte® to monitor spheroid formation is optional and alternatively can be performed using a microscope.

Day 3

3. Add T cells and treatments

a. Once spheroids have formed (72 h), remove the plate from the incubator and carefully add appropriate activators if necessary (e.g. CD3/CD28 Dynabeads) at 50 µL/well.
b. Prepare immune cells at an appropriate density. It is recommended that different target-to-effector ratios are tested (e.g., 1:3, 1:5).
c. Seed 50 µL/well immune cells on top of spheroids. Ensure well volume is at a total of 200 µL.
d. Place the assay plate back into the Incucyte® Live-Cell Analysis System. Continue scanning:
    a. Scan Type: Spheroid
    b. Channel Selection: Single spheroid, Phase + Brightfield and Green
    c. Objective: 4x
    d. Scan interval: 3 h
e. Assess spheroid killing and determine endpoint for iQue® assessment.
    Optional: Take 10uL supernatant samples at desired timepoints for temporal cytokine analysis.

4. Spheroid dissociation

4.1. Matrigel not required

4.1.1. Remove final 10 µL/well supernatant and place into a V-bottom plate for cytokine analysis if required. Option to freeze at -20 °C until analysis.

4.1.2. Gently remove 150 µL/well of supernatant and transfer to a V-bottom plate.

4.1.3. Add 150 µL/well PBS. Centrifuge 125 x g, 1 minute.

4.1.4. Remove 150 µL/well and add to the V-bottom plate.

4.1.5. Add 150 µL/well Accutase to ULA plate and incubate at 37 °C for 1 hour.

4.1.6. Centrifuge V-bottom plate 300 x g, 5 minutes. Flick and resuspend by shaking 2400 rpm, 1 minute. Place plate at 37 °C.

4.1.7. After 1 hr triturate ULA plate. Use a light microscope to examine spheroid dissociation. If cell clusters remain,

repeat incubation for a further 15 minutes and triturate. Repeat until a single cell suspension is achieved.

4.1.8. Transfer entire contents of wells to V-bottom plate. Add 100 µL/well PBS into the ULA plate to wash wells and transfer to the V-bottom. Centrifuge V-bottom 300 x g, 5 minutes.

4.1.9. Flick V-bottom plate to remove supernatant, resuspend cells in residual media by shaking 2400 rpm, 1 minute.

4.1.10. Label using antibody detection reagent and viability dye for 1 hour using volumes as per kit instructions.

4.2. Matrigel required

4.2.1. Follow steps 4.11 – 4.14

4.2.2. Add 150 µL/well of Cell Recovery Solution. Shake at 4 °C for 30 minutes (1000 rpm)

4.2.3. Remove 150 µL/well and add this to the V-bottom plate.

4.2.4. Add 150 µL/well Accutase to ULA plate. Shake at 4 °C for 1 hour (1000 rpm).

4.2.5. Centrifuge V-bottom plate 300 x g, 5 minutes. Flick and resuspend by shaking 2400 rpm, 1 minute. Place plate at 4 °C.

4.2.6. After 1 hr triturate ULA plate. Use a light microscope to examine spheroid dissociation. If cell clusters remain, repeat incubation for a further 15 minutes and triturate. Repeat until a single cell suspension is achieved.

4.2.7. Follow steps 4.1.8 – 4.1.9.

5. Cytokine analysis

5.1. Prepare cytokine standards as per kit instructions.

5.2. Add diluted cytokine capture beads and incubate for 1 hr (RT, dark)

5.3. Spin (300g, 5mins) and aspirate supernatant. Shake (3000 rpm, 60s) to resuspend.

5.4. Add cytokine detection cocktail and incubate for 2 hours (RT, dark).

5.5. Wash, spin and resuspend as per kit protocol.

5.6. Run on iQue® using template provided.

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