Detailed Immune Cell Killing Protocol for Adherent Target Cells

Detailed Demonstration Protocol

The following protocol is a detailed example designed to enable you to run a successful IncuCyte™ Immune Cell Killing Assay using adherent target cells. Note that the protocol does not include a description of any experiments required for optimizing seeding density or target: effector cell ratios. Here we specifically describe the use of the IncuCyte ZOOM® instrument for establishing and quantifying the killing of SK-OV-3 ovarian cancer cells by anti CD3 antibody/IL-2 activation of the T cell subpopulation of peripheral blood mononuclear cells (PBMCs).

Materials

- Target cells: SK-OV-3 ovarian cancer cells
  - Optional—tumor cells can be labeled with NucLight™ Red (Essen BioScience 4476) to provide a simultaneous count of viable target cells.
- Growth medium: McCoy’s 5a medium + 15% FBS + 1% Glutamax
- Effector cells: Peripheral blood mononuclear cells (fresh or frozen, e.g., Cambridge BioScience CTL-PBMC/SER-PBMC)
- 96-well flat-bottom microplate (Corning 3595)
- Assay medium: RPMI 1640 (Life Technologies 11875-085) + 10% FBS
- IncuCyte™ Caspase 3/7 Reagent (Essen BioScience 4440)
- Anti-CD3 antibody (eBioscience 16-0037) + rh IL-2 (PreproTech 200-02)
- 0.25% Trypsin/EDTA (Life Technologies 25200)
- D-PBS (w/o Ca²⁺, Mg²⁺; Life Technologies 10010)

Protocol

Day 0

1) Remove serum-containing medium from SKOV-3 target cell culture and gently rinse twice with D-PBS.
   **Note:** Culture should be at 75–80% confluence in a T-75 flask.
2) Harvest cells and perform a cell count (e.g., trypan blue staining + hemacytometer). Centrifuge the cell suspension (1000 RPM, 4 minutes) and resuspend the cell pellet in culture medium at 10,000 cells/mL.
3) Using a manual multichannel pipette, seed cells (100 µL/well, i.e., 2000 cells/well) into every well of a flat-bottom microplate.
4) Let the plate stand at ambient temperature for 30 min. Position the plate into the IncuCyte ZOOM® live-cell imager and leave for 20 min to equilibrate before scheduling the first scan.
5) Schedule 24-hr repeat scanning (10x objective) for every 3 hr, with the first scan to commence immediately. Monitor cell confluence for the next 18 hr (overnight) until the desired confluence (e.g., 20%) is achieved.

Day 1

1) On the morning of the assay prepare a 10 µM solution of the IncuCyte™ Caspase 3/7 apoptosis green fluorescence detection reagent (Essen BioScience 4440) (4x final assay concentration of 2.5 µM) in assay medium. Warm to 37°C before incubation.
2) Prepare the anti-CD3 antibody (100 ng/mL) + IL-2 (10 ng/mL) T cell activator treatment at 4x final assay concentration in assay medium and warm to 37°C.
3) Remove the target cell plate from the incubator. Aspirate the medium, taking care not to damage the cell layer. Using a multichannel pipette, transfer 25 µL of the warmed caspase 3/7 solution into each well. Then transfer 25 µL of the warmed anti-CD3 antibody + IL-2, or vehicle, into the appropriate wells of the cell plate.
4) Add an additional 50 µL medium containing the effector cells (PBMCs, see below) to form a total assay volume of 100 µL.
   **Note:** It is advised that some control wells containing only target cells are included (+ vehicle and + PBMC activators).
5) Remove any bubbles from all wells by gently squeezing a wash bottle (containing 100% ethanol with inner straw removed) to blow vapor over the surface of each well. Keep the tip of the wash bottle approximately 5 cm from the surface of the medium.
6) Position the de-bubbled cell plate in the IncuCyte ZOOM® instrument and allow it to equilibrate for 20 min prior to the first scan. Schedule 24-hr repeat scanning (10x) for every 2–3 hr for up to 5 days.
   a. Objective: 10x,
   b. Vessel Type: Corning 3595
   c. Scan Mode: Standard
   d. Scan Pattern: 2 images per well
   e. Channel: Phase + “Green” (+“Red” if NucLight™ Red target cells are used)
Preparation of PBMCs:
The target cell:PBMC ratio for this protocol is 1:5. It is recommended that more than one ratio is tested in order to observe optimum levels of target cell death.

1) If using frozen PBMCs, place cells in a water bath at 37°C and once thawed, remove the contents and gently transfer to a 50 mL Falcon tube. If using fresh PBMCs, remove the contents and gently transfer to a 50 mL Falcon tube.
2) Slowly add warmed assay medium to the cell suspension to make a total volume of 40 mL, gently agitating at the same time.

Analysis
Target-cell apoptosis is quantified in the IncuCyte™ software by counting the total number of “large” green-fluorescent objects (nuclei) in the field of view over time. Proliferation of target cells is measured from the red object count, corresponding to the number of red cell nuclei. Data are expressed as the number of fluorescent objects per mm². To generate these metrics, the user must create a Processing Definition and Analysis Job, as described within the IncuCyte ZOOM® Fluorescent Processing Overview Technical Note, which are suited to the cell type, assay conditions, and magnification. Note that caspase 3/7–positive effector cells will also fluoresce. Analysis filters (area, mean intensity, eccentricity) should be applied to exclude these cells (and thus isolate the target cell signal) from the analysis.

Example data generated with the Detailed Immune Cell Killing Protocol for Adherent Target Cells

T-cell killing. Representative blended phase contrast and green-fluorescent images (10x) of SK-OV-3 and PBMC co-cultures in the presence of IncuCyte™ Caspase 3/7 Reagent at 96 hr in non-activated PBMCs (control; A, B) and activated PBMCs (anti-CD3 antibody [100 ng/mL] + IL-2 [10 ng/mL]) (C, D). In each case, the right-hand panel shows the masking (yellow) of apoptotic target (but not effector) cells with the IncuCyte™ processing definition. Note the induction of target-cell apoptosis in the anti-CD3 antibody/IL-2–treated co-cultures. Panel (E) shows an IncuCyte™ software view of the time-course of target-cell caspase 3/7 apoptotic cell (green object) count with non-activated PBMCs (blue) and activated PBMCs (anti-CD3 antibody [100 ng/mL] + IL-2 [10 ng/mL]) (pink) co-cultures. Note the onset of apoptosis in the treatment group at approximately 20 hr.
**T-cell killing and inhibition of proliferation.** Representative blended phase contrast and red-fluorescent images (10x) of NucLight™ Red labeled SK-OV-3 and PBMC co-cultures in the presence of IncuCyte™ Caspase 3/7 substrate at 96 hr in non-activated PBMCs (control; A, B) and activated PBMCs (anti-CD3 antibody [100 ng/mL] + IL-2 [10 ng/mL]) (C, D). In each case, the right-hand panel shows the masking of target-cell nuclei (blue) with the IncuCyte™ processing definition. Note the inhibition of target-cell proliferation in the anti-CD3 antibody/IL-2–treated co-cultures. Panel (E) shows an IncuCyte™ software view of the time-course of target-cell proliferation (red object count) with non-activated PBMCs (blue) and activated PBMCs (anti-CD3 antibody [100 ng/mL] + IL-2 [10 ng/mL]) (pink) co-cultures. Note the onset inhibition of proliferation at approximately 20 hr.