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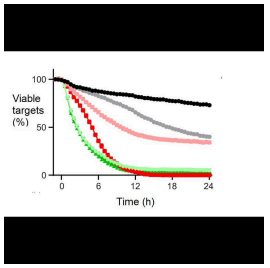
# Time-lapse killing assay (monolayer - IncuCyte) V.2

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**Protocol status:** Working
**We use this protocol and it's working**

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## Abstract

Fluorescent target cells are plated in a monolayer in a 96 well plate. Effector cells are added to that plate and time-lapse imaging in combination with fluorescent indicators of cell death reveal the dynamics of target cell death.

## Guidelines

To obtain an even monolayer, mix cell suspensions well before adding them into the plate. It may be necessary to agitate the reservoir to ensure the best suspension possible. Add cells gently using a multichannel pipette with the plate on a flat surface. Once cells are plated, leave the plate undisturbed for 10 min at room temperature to ensure an even monolayer adheres to the plastic.

To avoid disturbing the monolayer when adding effector cells, dribble cells down the edge of the well, angling the pipette tip towards the edge of the well.

To reduce costs, target cell survival can be tracked using Nuclight Red fluorescence alone (without an indicator of apoptosis) since these cells lose fluorescence when they die.

Important controls include target alone wells, targets with drugs and no effectors, targets with effectors and no drugs.

***Common pitfalls:***

**Variation between replicates wells**

Diagnosis: a large standard deviation in live targets per well in the first image. Inconsistent increase in image confluence between the first image and when effectors were added.
Fix: If target cell variation is a problem increase the number of replicate wells until technique improves. For effector cell variation, a 96 well 'master plate' containing all effectors and drugs to be added will reduce the time between the first effectors being added and the last effectors being added to the target cells across multiple conditions.

**Condensation, air bubbles or marking on the plastic impair target cell counts.**

Diagnosis: single replicates have sudden changes in target cell number or blurry images are apparent in initial frames.
Fix: Bubbles in the well, condensation on the lid or markings on the plastic within the light path will reduce the quality of the images. Bubbles can be popped using a wash bottle containing 70% ethanol with the tube removed to prevent ethanol liquid leaving the bottle. Ethanol vapors will pop the bottle when gently blown onto the meniscus layer. Do not mark plates and pre-warm them in the incubator for 15 min prior to imaging to avoid condensation and marks on the plastic impairing the images.

**Effector cells are added too harshly and disturb the target cell monolayer.**

Diagnosis: target cell counts drop dramatically between first image and the addition of effectors with distinct zones of the image that are clearly effected.
Fix: check that poly-L-ornithine is working effectively. Alter technique for addition of effectors. Normalize to the first image after effectors are added to avoid spuriously implying effector cells are killing target cells that have been knocked out of the field of view.

**Tumor alone condition becomes confluent impacting the apparent rate of cytotoxicity normalized to tumor alone controls.**

Diagnosis: target cell numbers in control wells plateau.
Fix: reduce the number of target cells per well to ensure they reach confluence at the end of the full assay, not before.

**Improper masking**
Diagnosis: target cell numbers decrease over time in the tumor alone condition.
Fix: Drop the threshold of detection for target cell masks if using amine reactive dyes to ensure target cells can be detected over time after they have divided. Size thresholds on the overlap masks (target cells+ caspase 3/7+) will ensure small dead effectors (caspase 3/7+) floating above live target cells do not get counted as dead target cells.

## Troubleshooting

## Before start

If using adherent target cells, label them and plate them the day before the assay.

If using non-adherent target cells, the day of the assay, start by pre-coating a plate with poly-L-ornithine.

## Overview

1 Target cells are fluorescently labelled to differentiate them from effector cells.

1.1 *Option 1:* Target cells are labeled using an amine-reactive dye (CellTrace Far Red Proliferation Kit, Cat. No:C34564, Thermo Fisher) according to the manufacturer's instructions.

1.2 *Option 2:* Target cells are stably transduced with a red fluorescent protein (IncuCyte NucLight Red Lentiviral Reagent - EF1α Puro, Cat. No. 4625, Essen Bioscience) according to the manufacturer's instructions.

1.3 *Option 3:* Target cells are stably transfected with a green fluorescent protein (GFP). In this situation, a red cell death indicator should be used (IncuCyte Caspase 3/7 Red Apoptosis Assay Reagent, Cat. No. 4704, Essen BioScience).

2 Labelled target cells are plated in a monolayer in a 96 well flat-bottom plate (Cat. No: 353072, Corning). Adherent targets are allowed to adhere overnight. Non-adherent target cells can be immobilised on plates pre-coated with 0.01% poly-L-ornithine (pre-coated for 2 h at room temperature; Cat. No. A-004-C, Fisher Scientific). Leave plates untouched for 10 min at room temperature after plating to ensure an even monolayer.

Cell numbers are titrated for each target, but are generally set at $2 \times 10^4$ cells/well for suspension cells and $1 \times 10^4$ cells/well for adherent cells in 100 µL.

3 *Optional:* An indicator of cell death is added to the media (1/1000 dilution; IncuCyte Caspase-3/7 Green Apoptosis Assay reagent, Cat. No:4440, Essen BioScience).

4 Take a 'targets alone' image prior to addition of effectors. Live targets per well are normalized using these values.

5 Enriched NK cells and any required drugs are added to the target cell monolayer at a 2:1 effector:target ratio, bringing the final volume to 200 µL.

*Depending on the sensitivity of the target cells to the various treatments and natural cytotoxicity, different effector:target ratios may be required for optimal sensitivity of the assay.*

6 Plates are placed in an incubator equipped with a time-lapse microscope with 4x, 10x and 20x lenses (IncuCyte Zoom or S3, Sartorius Inc.) for 48 hours. Images are recorded at 30 min intervals.

7 Image analysis is performed in the Incucyte software. Live targets (Target cell fluorescence+ Caspase3/7-) are detected at each time point. The percentage of live targets at any given time point is normalized for the number of live targets in each well at the starting time point (t = 0 h), and further normalized to the growth of targets growing alone. This data is then plotted in GraphPad Prism.