



## CyToxiLux™

### Overview:

CyToxiLux™ is OncoImmunitin, Inc.'s single cell-based fluorogenic cytotoxicity assay. The advantages of this assay (**Nature Med.** 8:185-189 (2002)) over others, e.g., <sup>51</sup>Cr release, are: (1) cytotoxicity is measured as a fundamental biochemical pathway leading to cell death (cleavage of a cell permeable fluorogenic caspase substrate) rather than merely as the end result of cell lysis, (2) in several systems tested, CyToxiLux™ was more sensitive (could detect relatively weak CTL responses against subdominant epitopes) and more rapid, (3) cell death can be measured exclusively in target cell populations by flow cytometry or fluorescence microscopy, and (4) when combined with immunophenotypic analyses and multiparameter flow cytometry, CTL-mediated killing of primary host target cells as well as the physiology and fate of effector cells can be directly visualized and monitored.

**Target cells** are fluorescently labeled (red) and then coincubated with cytotoxic effector cells. At the desired time point, medium is removed from samples and replaced with a solution containing a fluorogenic **caspase substrate**. Following incubation and washing, samples may be analyzed by flow cytometry or fluorescence microscopy. Cleavage of the **substrate** results in increased green fluorescence in dying cells.

**Please read this entire protocol before commencing assay!**

#### Components supplied in CyToxiLux™ kit

**Vial CS** (x3) = **Caspase Substrate** solution

**Vial T** (x1) = **Target cell** marker

**Staining Buffer** bottle (x1)

#### Components supplied by user

Effector cells

**Target cells**

Assay Medium

**Medium A** = Assay Medium. Medium in which assay will be run, *i.e.*, medium in which Target and Effector cells will be coincubated.

**Medium T** = Target Cell Medium. **Medium A** plus **Target cell** marker. This is prepared by adding 1 µl from **Vial T** per ml of **Medium A**. (**Target cell** marker is not CTO™, the probe used in the **Nature Med.** reference above; rather, it is a dye with fluorescence properties more complementary to those of the **caspase substrate**.) Please read entire protocol in order to determine total volumes.

**Note:** The assay may be performed using either **96-well plates** or polypropylene **microcentrifuge tubes**. Microcentrifuge tubes are recommended for **Target cells** which adhere in culture, as re-adhesion to the 96 well plate during co-incubation with Effector cells can result in sample loss.

**Washing** is defined as centrifugation followed by careful removal of all liquid from wells or tubes. Resuspension of pellets should be done with gentle pipetting of plates or tapping of tubes with finger. **DO NOT VORTEX.**

### Preparation of **Target cells**

1. Suspend **Target cells** (suspension cells or trypsinized adherent cultures) in medium from **Vial T** at  $2 \times 10^6$  cells/ml. If experimental design includes pulsing with sensitizers, e.g., peptides, they should be added to the appropriately sized Effector cell aliquots at this stage.
2. Incubate at 37°C for 1 hour. During this 1 hour, prepare Effector cells (see below).
3. Add at least a 10-fold volume of **Medium A** and wash. Repeat twice.
4. Resuspend labeled **Target cells** at  $2 \times 10^6$  cells/ml in **Medium A**.
5. Dispense 100 µl of **Target cell** suspension to each assay well or tube.

### Preparation of Effector cells

1. Prepare Effector cells at the appropriate concentration in **Medium A**. For example, for a final Effector to **Target** ratio of 25:1, prepare Effector cells at  $5 \times 10^7$  cells/ml.

### Coincubation of **Target** and Effector cells

1. Add 100 µl of Effector cell suspension to each well containing **Target cells** except at least two wells, and add 100 µl of Effector cell suspension to at least two wells which do not contain **Target cells**.
2. Add 100 µl of **Medium A** to the wells containing only **Targets** or only Effectors to bring all samples to a final volume of 200µl.
3. Coincubate for the appropriate time in the appropriate 37°C environment, *i.e.*, for a CO<sub>2</sub>-containing medium,

place in a CO<sub>2</sub>-containing incubator. We recommend 1-3 hours but the exact time will depend on the cells of interest. Since this assay detects dying cells rather than cell lysis, incubation times for a given cell system should be significantly shorter than with the <sup>51</sup>Cr release methodology.

4. Wash samples and resuspend one well containing **Target cells** only and one well containing Effector cells only with 75 μl of Staining Buffer. To all other samples add 75 μl of **substrate** from **Vial CS**.
5. Incubate at 37°C for 30-60 minutes.
6. Wash twice with **Staining Buffer**.
7. Resuspend in **Staining Buffer**, transfer samples to flow cytometry tubes and analyze by flow cytometry.

**Summary of samples:**

- Target cells**
- Target cells + Substrate** from **Vial CS**
- Target cells + Effector cells + Substrate** from **Vial CS** (multiple samples)
- Effector cells
- Effector cells + **Substrate** from **Vial CS**

**Flow Cytometry:**

1. Use sample **A** to initially set **FL1** and **FL2** channels. Place the peak for cells from sample **A** between 10<sup>1</sup> and 10<sup>2</sup> in the **FL1** channel near 10<sup>3</sup> in the **FL2** channel.
2. Use sample **E** to setup **FL2** compensation. Dead/dying Effector cells may show a high **FL1** x **FL2** population on most single-laser flow cytometers. Compensate **FL2** by **FL1** until this population is on the same horizontal axis as viable Effector cells (low **FL2**).
3. Use sample **B** to setup compensation of the **FL1** channel.
4. Run remaining samples

**Sample flow cytometric data:**

**Target cells** (Jurkat, K562, or MDA-MB-468) were incubated with or without Effector cells (NK-92, 5:1 Effector:Target ratio) for 1 hour at 37°C followed by a 45 minute incubation with the **caspace substrate**. Quadrants **R1** (upper left of each panel) represent viable **target cells** while quadrants **R2** (upper right) represent dying, **substrate**-positive **target cells**. Effector cells occupy the lower 2 quadrants. The percent live and dead **target cells** (inset % values) is calculated as **R1/(R1+R2)** or **R2/(R1+R2)**, respectively. All cell lines were purchased from ATCC.

