



## CyToxiLux® PLUS!

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### Overview

**CyToxiLux® PLUS!** is OncoImmunitin, Inc.'s single cell-based fluorogenic cytotoxicity kit for the measurement of downstream caspase, specifically caspase 6, activity in live target cells following the successful delivery of a lethal hit by cytotoxic lymphocytes. Whereas previous **CyToxiLux®** kits were formulated for 50 assays with single (488 nm) or dual (488 and 633 nm) laser flow cytometers, the current **CyToxiLux® PLUS!** kit is formulated for 80 assays with dual laser cytometry capability. (For single laser systems, please contact OncoImmunitin, Inc. directly.)

**CyToxiLux® PLUS!** is similar to **GranToxiLux® PLUS!** and **PanToxiLux™**, OncoImmunitin, Inc.'s other single cell cytotoxicity assay kits, with the difference being the cell permeable, fluorogenic substrate: **CyToxiLux® PLUS!** is designed to detect downstream caspase activity only, **GranToxiLux® PLUS!** granzyme B activity, and **PanToxiLux™** detects *both* granzyme B and an upstream caspase, specifically caspase 8, activities. As with the other two kits, **CyToxiLux® PLUS!** can be used for selection of antibodies operating *via* an antibody-dependent cellular cytotoxicity (ADCC) mechanism in both low and high throughput screening (HTS) modes.

Advantages of **CyToxiLux®**, **GranToxiLux®**, and **PanToxiLux™** over other cytotoxicity assays, e.g., <sup>51</sup>Cr release, LDH release, and PI, include: (1) cytotoxicity is measured as a fundamental biochemical pathway leading to cell death (cleavage of a cell permeable fluorogenic substrate) rather than merely as the loss of plasma membrane permeability and its sequelae, (2) sensitivity is enhanced such that relatively weak CTL responses against subdominant epitopes are detectable (3) rapidity (Effector:Target coincubation times between 0.3 and 2 hours), (4) measurement of cell death can be carried out exclusively in target cell populations by flow cytometry or fluorescence microscopy, and (5) when combined with immunophenotypic analyses and multiparameter flow cytometry, cytotoxic lymphocyte-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 in the presence of a fluorogenic **granzyme B substrate**. Following incubation and washing, samples may be analyzed by flow cytometry. Cleavage of the **substrate** results in increased green fluorescence in dying cells. Real-time imaging can also be carried out with confocal microscopy.

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

#### Components supplied in **CyToxiLux®** kit (sufficient for 80 assays)

**Vial CS** (4 vials) = **Caspase Substrate** solution

**Vial TFL4** (1 vial) = **Target cell** marker for use with dual laser instruments

TFL dilution medium (1 small Eppendorf tube) = Resuspension medium

For **Vial TFL4**

#### Components supplied by user

Effector cells

Target cells

**Wash Buffer** bottle (1 bottle)

In order to eliminate nonviable cells from the starting target cell population, e.g., due to a freeze/thaw cycle, please contact OncoImmunitin, Inc. Please note: labeling of cell surfaces with fluorescent probes is **not** recommended as this may interfere with effector-target interactions. When used according to the procedure herein, TFL4 does not label cell surfaces.

**Format:** The assay may be performed using either 96-well plates or polypropylene microcentrifuge tubes.

**Reconstitution of TFL4:** Add 25 µl from the TFL dilution medium tube to **Vial TFL4**. (Once reconstituted, **TFL4** should be stored at -20°C.)

**Medium T** = Medium for labeling target cells. 1 µl from reconstituted **TFL4** is added to the medium in which target cells had been grown or to a physiologic buffer such as phosphate buffered saline (PBS). Please note: most cells load more efficiently in PBS or in a medium free of serum. If 10% serum is included, the recommended **TFL4** dilution is 1:1000 whereas for serum-free buffers, the **Target cell** marker is typically used at 1:3000; however, further dilution may be superior. *Optimal TFL4 concentrations should be determined for individual target cell types as 1:1000 for serum-containing and 1:3000 for serum-free media are merely suggested starting points.*

**Washing** is defined as addition of the indicated volume of medium/buffer followed by centrifugation and then careful removal of all liquid from tubes or flicking followed by light tapping of plates. Resuspension of pellets should be carried out with gentle pipetting and/or tapping of tubes with finger. **DO NOT VORTEX.**

## Protocol

### Preparation of Target cells

1. Suspend Target cells (suspension or trypsinized adherent cells) in **Medium T** at  $2 \times 10^6$  cells/ml. (This is a suggested concentration. Lower numbers can and are routinely used. The critical point is to be able to collect 2,000-10,000 Target cells for analysis (*vide infra*.) If targets are to be pulsed with sensitizers, e.g., peptides, the latter should be added at this stage. (If peptide solubility requires use of an organic cosolvent, the latter should not exceed 0.3% (v/v) and a vehicle control tube/well should be included.) Incubate at 37°C for 0.25-1.0 hour. Optimal time should be determined for individual cell types (and sensitizers). Please note: for loading of PBLs, a 1:1000 dilution of **TFL4** in PBS for 15' at 37 °C is recommended. If peptides are to be used with targets, the recommendation is to add **TFL4** for the final 15' of sensitizer exposure.
2. During this time, prepare Effector cells (see below).
3. Wash **Target cells** 2 times with at least a 10-fold excess volume of physiologic buffer/medium per wash.
4. Count labeled **Target cells**. (It is essential to count cells at this point as there is always cell loss with washing.) Resuspend labeled **Target cells** at  $1 \times 10^6$  cells/ml in **Wash Buffer**. (Depending on the experimental design, lower numbers of **Target cells** may be used.  $1 \times 10^6$  cells/ml is recommended for cases in which  $1 \times 10^5$  Targets/well or tube will be used.)
5. Dispense 100  $\mu$ l of **Target cell** suspension to each assay well or tube.

### Preparation of Effector cells

1. Prepare Effector cells at the appropriate concentration in **Wash Buffer**. For example, for a final Effector to **Target** ratio of 5:1 where  $1 \times 10^5$  Targets/well or tube will be used, suspend Effector cells at  $5 \times 10^6$  cells/ml.

### Coincubation of Target and Effector cells

1. Add 100  $\mu$ l of Effector cell suspension to each well or tube containing **Target cells** except at least two wells, and add 100  $\mu$ l of Effector cell suspension to at least two wells which do not contain **Target cells**.
2. Add 100  $\mu$ l of **Wash Buffer** to wells containing only **Targets** and only Effectors to bring all samples to a final volume of 200  $\mu$ l.
3. Centrifuge all samples, carefully remove medium, and resuspend cell pellets in either 75 $\mu$ l **Substrate** from **Vial CS** or **Wash Buffer** for controls (absence of **Substrate** (for Tubes A and C below)). **For ADCC antibodies can be added at this time.**
4. Immediately after resuspension, pellet cells by brief centrifugation (Once the centrifuge reaches the speed normally used for cells, hold for 30 seconds and then stop the instrument.).
5. Incubate at 37°C for the desired time points. Since this assay detects dying cells rather than cells with irreversibly damaged plasma membranes, incubation times (as well as E:T) for a given cell system should be significantly shorter (and lower) than with other methodologies. For single point assays, the suggested coincubation time is 1 hour (enduser times typically range between 30 minutes and 2 hours). Substantially longer times are not recommended.
6. Wash each sample with 200  $\mu$ l **Wash Buffer**.
7. Resuspend each sample in **Wash Buffer**, transfer to flow cytometry tubes or leave in plates if a plate reader is to be used, and analyze by flow cytometry.

### Summary of samples:

- A. **Target cells** (=Target cells **TFL4**)
- B. **Target cells** + **Substrate** from **Vial CS**
- C. Effector cells
- D. Effector cells + **Substrate** from **Vial CS**
- E. **Target cells** + Effector cells + **Substrate** from **Vial CS** (multiple samples)

**Flow Cytometry: DO NOT VORTEX.** (*N.B.:* As this assay measures the delivery of a **lethal hit** to a **Target cell**, vortexing is strongly not advised.)

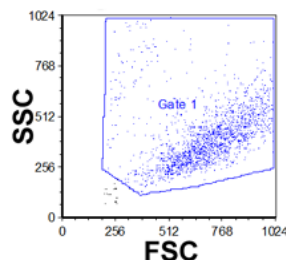
Channel settings consistent with the following excitation and emission peaks should be used:

**TFL4**  $\lambda_{ex}$ : 633 nm,  $\lambda_{em}$ : 657 nm

**CS**  $\lambda_{ex}$ : 488 nm,  $\lambda_{em}$ : 520 nm

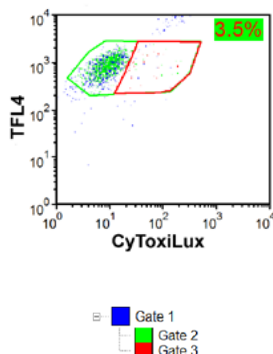
For instruments where software is set for specific probes: **APC** for **TFL4** and **FITC** for **CS**.

1. On a dot plot of FSC (x-axis) vs. SSC (y-axis), place **Targets cells** (sample A) in the low right and draw **Gate 1** as shown below (Use of this gate is to delete acellular events):

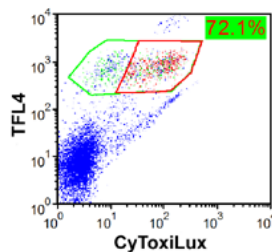


2. Use samples **A** and **D** to initially set the **APC** (for **TFL4**) and **FITC** (for **CyToxiLux**<sup>®</sup>) channels, resp.: place the peak for cells from sample **A** near  $10^3$  in the **APC** channel and the peak from sample **D** at *ca.*  $10^1$  in the **FITC** channel. Cells in

sample B should then be at *ca.*  $10^3$  in the APC and  $10^1$  in the FITC channels, as shown below. Optimization can be carried out with small PMT adjustments with sample B. (Note: Healthy (>95% viable by Trypan Blue exclusion) TFL4-labeled Target cells should appear as a single population. If not, first try decreasing labeling time to 15 minutes. If more than one population still exists, decrease TFL4 concentration.)



- Run remaining samples. 2,000-10,000 Target cells per sample should be collected for analysis into Gate 2 (in figures above and below). Gate 2 should be nested inside Gate 1. In the example below in which the E:T (NK92:K562) is 4:1, the percent of target cells capable of cleaving the CyToxiLux<sup>®</sup> substrate is 72.1%. This compares with the 3.5% background of target cells alone (shown in figure above). Please note: this background can be decreased significantly by use of NFL1, a probe available from OncoImmunit, Inc. requiring a cytometer with a PacBlue channel (excitation of *ca.* 405 nm and emission of *ca.* 465 nm).



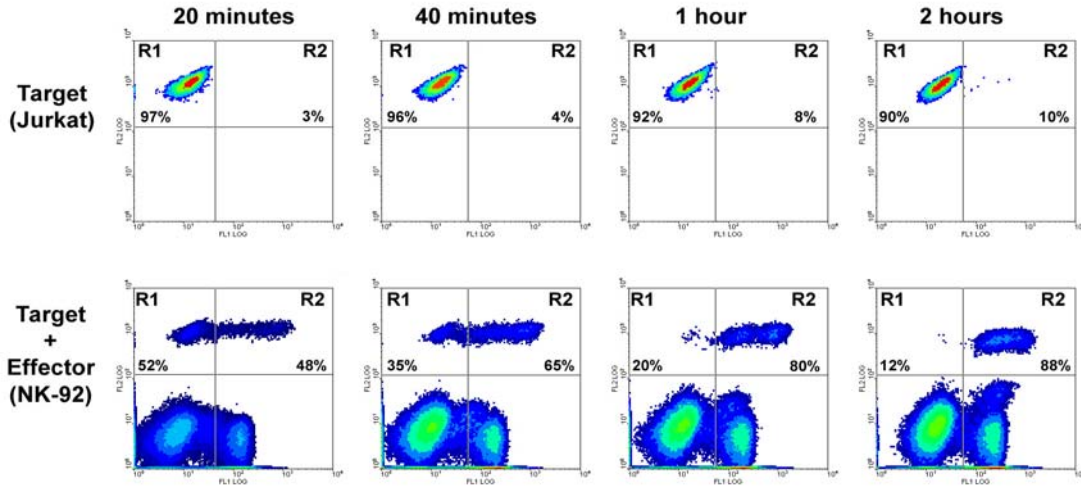
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### Sample Flow Cytometry Data

All cell lines were purchased from the ATCC.

**Target cells** (Jurkat) were incubated in 75  $\mu$ l **Caspase Substrate** with or without Effectors (NK-92) at a 5:1 Effector:**Target** ratio for the indicated times. Quadrants **R1** (upper left of each panel) represent nonapoptotic target cells while quadrants **R2** (upper right) represent apoptotic, **Substrate**-positive target cells. Effector cells occupy the lower 2 quadrants in Effector + Target samples. The percent nonapoptotic and apoptotic target cells (inset % value) is calculated as  $R1/(R1+R2)$  or  $R2/(R1+R2)$ , respectively.



**Target cells** (Jurkat, K562, or MDA-MB-468) were incubated with or without Effectors (NK-92) at a 5:1 Effector:**Target** ratio for 1 hour at 37°C with the **Caspase Substrate**. Quadrants **R1** (upper left of each panel) represent nonapoptotic target cells while quadrants **R2** (upper right) represent apoptotic, **Substrate**-positive target cells. Effector cells occupy the lower 2 quadrants. The percent nonapoptotic and apoptotic target cells (inset % value) is calculated as  $R1/(R1+R2)$  or  $R2/(R1+R2)$ , respectively.

