



CyToxiLux® PLUS!
(A new, improved version of CyToxiLux®)
Updated 08/12/04

Overview

CyToxiLux® PLUS! 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., ⁵¹Cr 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 in the presence of a fluorogenic **caspase 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® PLUS! kit (sufficient for 50 assays)

Vial CS (3 vials) = **Caspase Substrate** solution
Vial TFL2 (1 vial) = **Target cell** marker for use with single laser instruments
Vial TFL4 (1 vial) = **Target cell** marker for use with dual laser instruments
T medium (2 small pink Eppendorf tubes) = Resuspension medium
for **Vials TFL2 and TFL4**
Wash Buffer bottle (1 bottle)

Components supplied by user

Effector cells
Target cells
Assay/Culture medium

Format: 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 a 96-well plate during co-incubation with Effector cells can result in sample loss.

Reconstitution of TFL2 or TFL4: Add 25 µl from one of the **T medium** tubes to **Vial TFL2 or Vial TFL4**. (Once reconstituted, **Vial TFL2 or TFL4** should be stored at -20°C.) **TFL2** is for use with flow cytometers equipped with only Ar ion (488 nm) lasers; measurements require compensation (please see **FLOW CYTOMETRY** instructions below). **TFL4** is for use with flow cytometers equipped with both Ar ion (488 nm) and red He-Ne (633 nm) lasers; measurements do not require compensation. (Neither **Target cell** marker is CTO™, the probe used in the **Nature Med.** reference above; rather, they are dyes with fluorescence properties more complementary to those of the **Caspase Substrate**.)

Medium A = Growth Medium for target cells.

Medium T = Medium for labeling target cells = **Medium A** plus **Target cell** marker. 1 µl from either reconstituted **TFL2** or **TFL4** is added per ml of **Medium A**. Please note: most cells load more efficiently in PBS or in a medium free of serum. If serum is excluded, the **Target cell** marker is typically used at ca. one third the concentration, *i.e.*, 1 µl from **Vial TFL2** or **TFL4** diluted in 3 ml of PBS or a serum-free medium; however, further dilution may also be possible. Optimal **TFL2** or **TFL4** concentrations should be determined for individual target cell types as 1:1000 for serum-containing media 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 wells or tubes. Resuspension of pellets should be carried out with gentle pipetting of plates or tapping of tubes with finger. **DO NOT VORTEX.**

Rapid Protocol

Preparation of Target cells

1. Suspend Target cells (suspension or trypsinized adherent cells) in **Medium T** at 2x10⁶ cells/ml. If experimental design includes pulsing with sensitizers, e.g., peptides, they should be added at this stage. (If peptide solubility requires use of an organic cosolvent, the latter should not exceed 0.5 % (v/v) and a vehicle control tube/well should be included.)
2. Incubate at 37°C for 0.5-1.0 hour. Optimal time should be determined for the individual cell types and sensitizers. During this time, prepare Effector cells (see below).
3. Wash **Target cells** at least 1x (some cell types require additional washes) with at least a 10-fold volume of **Medium A**.
4. Resuspend labeled **Target cells** at 2x10⁶ 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, suspend Effector cells at 5×10^7 cells/ml.

Coincubation of **Target** and Effector cells

1. Add 100 μ l of Effector cell suspension to each well (or tube) containing **Target cells** except at least two wells, and add 100 μ l of Effector cell suspension to at least two wells which do not contain **Target cells**.
2. Add 100 μ l of **Medium A** to wells containing only **Targets** and only Effectors to bring all samples to a final volume of 200 μ l.
3. Centrifuge all samples, carefully remove medium, and resuspend cell pellets in either 75 μ l **Substrate** from **Vial CS** or **Medium A** for controls (absence of **Substrate** (for Tubes A and C below)).
4. Immediately after resuspension, pellet cells by brief, ca. 1 minute, centrifugation.
5. Incubate at 37°C for the desired time points. Since this assay detects dying cells rather than cell lysis, incubation times for a given cell system should be significantly shorter than with the ^{51}Cr release methodology. Typical coincubations times range between 20 minutes and 2 hours.
6. Wash samples with 200 μ l **Wash Buffer**.
7. Resuspend in **Wash Buffer**, transfer to flow cytometry tubes, and analyze by flow cytometry.

Summary of samples:

- A. **Target cells**
- 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:

For flow cytometers equipped with both Ar ion (488 nm) and red He-Ne (633 nm) lasers (**TFL4** is used as target cell dye):

1. Use samples **A** and **D** to initially set the **FL4** and **FL1** channels, resp.: place the peak for cells from sample **A** near 10^3 in the **FL4** channel and the peak from sample **D** at ca. 10^1 in the **FL1** channel. Cells in sample **B** should then be at ca. 10^3 in the **FL4** and 10^1 in the **FL1** channels.
2. Run remaining samples. 10,000-20,000 **Target cells** per sample should be collected for analysis.

For flow cytometers equipped with only an Ar ion (488 nm) laser (**TFL2** is used as target cell dye):

1. Use samples **A** and **D** to initially set the **FL2** or **FL3** and **FL1** channels, resp. Place the peak from sample **A** near 10^3 in the **FL2** or **FL3** channel (**FL3** is often superior to **FL2** (less bleed-through with adequate intensity) and should be considered at this point.) and the peak from sample **D** at ca. 10^1 in the **FL1** channel.
2. Use sample **B** to set up **FL2/FL3** compensation. Dead/dying Effector cells may show a high **FL1** x **FL2/FL3** population on most single-laser flow cytometers. Compensate **FL2/FL3** by **FL1** until this population is on the same horizontal axis as viable Effector cells (low **FL2/FL3**).
3. Run remaining samples. 10,000-20,000 **Target cells** per sample should be collected for analysis.

Standard Protocol

As an alternative to the Rapid Protocol described above, the following can be used, particularly for cells requiring a specific, e.g., growth, medium for effector:target incubations.

Preparation of **Target** and Effector cells

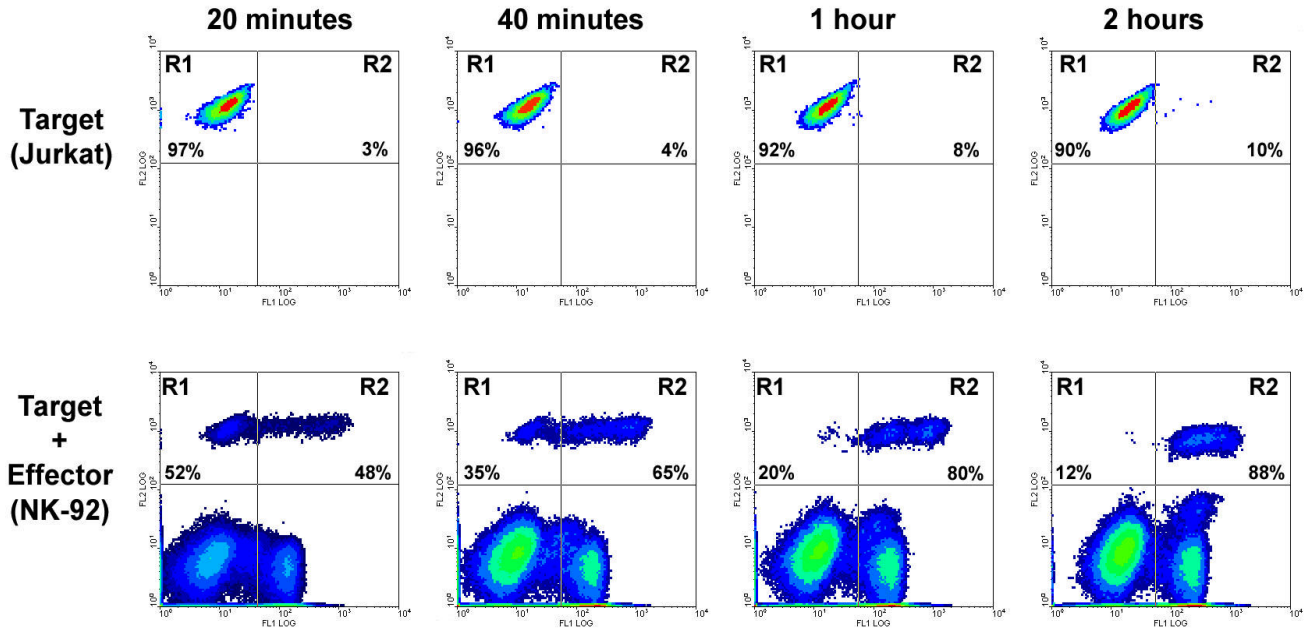
1. Prepare both **Target** and Effector cells as described in the Rapid Protocol.

Coincubation of **Target** and Effector cells

1. Add 100 μ l of Effector cell suspension to each well (or tube) containing **Target cells** except at least two wells, and add 100 μ l of Effector cell suspension to at least two wells which do not contain **Target cells**.
2. Add 100 μ l of **Medium A** to wells containing only **Targets** and to wells containing only Effectors to bring all samples to a final volume of 200 μ l.
3. Coincubate for the desired time in the appropriate 37°C environment, e.g., for a CO₂-containing medium, place in a CO₂-containing incubator. Incubation of 1-3 hours is recommended 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 ^{51}Cr release methodology.
4. Wash samples and resuspend one well containing **Target cells** only and one well containing Effector cells only in 75 μ l of **Wash Buffer**. To all other samples add 75 μ l of **substrate** from **Vial CS**.
4. Incubate at 37°C for 30-60 minutes.
5. Wash samples with 200 μ l **Wash Buffer**.
6. Resuspend in **Wash Buffer**, transfer samples to flow cytometry tubes and analyze by flow cytometry.
7. Follow Flow Cytometry instructions described above for Rapid Protocol.

Sample Flow Cytometry Data
All cell lines were purchased from the ATCC.

Target cells (Jurkat) were incubated in 75 μ l **Caspase Substrate** with or without Effector cells (NK-92) at a 5:1 Effector:**Target** ratio for the indicated times (Rapid Protocol). 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 in Effector + Target samples. The percent live and dying 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 Effector cells (NK-92) at a 5:1 Effector:**Target** ratio for 1 hour at 37°C followed by a 45 minute incubation with the **Caspase Substrate** (Standard Protocol). 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 dying target cells (inset % value) is calculated as $R1/(R1+R2)$ or $R2/(R1+R2)$, respectively.

