OncoImmunin, Inc.

GranToxiLux®-G_2D_2

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Overview

GranToxiLux® is OncoImmunin, Inc.'s single cell-based fluorogenic cytotoxicity assay for the measurement of granzyme B activity in live cells. This kit is similar to CyToxiLux®, OncoImmunin, Inc.'s other single cell cytotoxicity assay kit, with the difference being substitution of a cell permeable, fluorogenic substrate for the detection of granzyme B activity in the GranToxiLux® kit for a caspase substrate in the CyToxiLux® kit.

Granzyme B exists in lysosomal granules in an inactive form in effector, e.g., CTL and NK, cells. When degranulation of effectors is induced in the presence of target cells, Granzyme B as well as other granule contents such as perforin are taken up by the latter with Granzyme B believed to be the first active enzyme delivered to target cells. Thus, measurement of granzyme B activity inside target cells provides an extremely early quantitative assessment of cell-mediated cellular cytotoxicity. Additionally, GranToxiLux® as well as CyToxiLux® can be used for selection in both low and high throughput screening (HTS) modes of antibodies operating *via* an antibody-dependent cellular cytotoxicity (ADCC) mechanism.

Advantages of the GranToxiLux® and CyToxiLux® assay kits over other cytotoxicity assays, e.g., ^{51}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 sequalae, (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 measured exclusively in target cell populations by flow cytometry or fluorescence microscopy, and (5) 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 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 GranToxiLux® kit (sufficient for 50 assays)
Vial G_2D_2 (3 vials) = Granzyme B Substrate solution
Vial TFL4 (1 vial)= Target cell marker for use with dual laser instruments
TFL dilution medium (2 small Eppendorf tubes)=Resuspension medium TFL4
Wash Buffer bottle (1 bottle)

Components supplied by user
Effector cells
Target cells

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

Reconstitution of TFL4: Add 25 μl from one of the TFL dilution medium tubes to **Vial TFL4.** (Once reconstituted, **Vial TFL4** should be stored at –20°C.) TFL4 is for use with flow cytometers equipped with both green lasers, e.g., 488 nm or 532 nm lines, and a far red, e.g., 633 or 635 nm lasers; measurements do not require compensation.

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 tamping of plates. Resuspension of pellets should be carried out with gentle pipetting of 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 $2x10^6$ cells/ml. (This is a suggested concentration. Lower numbers can and are routinely used. The critical point is to be able to collect 5,000-10,000 Target cells for analysis.) 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.)

2. 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, 1:1000 dilution of TFL4 in PBS for 15' at 37 °C is recommended. If sensitizers are to be used with PBLs, the recommendation is to add TFL4 for the final 15' of sensitizer exposure.
3. During this time, prepare Effector cells (see below).
4. Wash Target cells 2 times with at least a 10-fold excess volume of physiologic buffer/medium per wash.
5. Resuspend labeled Target cells at $2x10^6$ cells/ml in **Wash Buffer**. (Depending on the experimental design, lower numbers of Target cells may used.)
6. 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 **Wash Buffer**. For example, for a final Effector to Target ratio of 10:1, suspend Effector cells at $2x10^7$ cells/ml.

Coincubation of Target and Effector cells

1. Add 100 µl of Effector cell suspension to each well (or tube) 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 **Wash Buffer** to wells containing only Targets and only Effectors to bring all samples to a final volume of 200 µl.
3. Centrifuge all samples, carefully remove medium, and resuspend cell pellets in either 75µl Substrate from Vial G_2D_2 or **Wash Buffer** for controls (absence of Substrate (for Tubes A and C below)). For ADCC antibodies should be added at this time.
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 cells with irreversibly damaged plasma membranes, incubation times for a given cell system should be significantly shorter than with other methodologies. Typical coincubations times range between 30 minutes and 2 hours. Longer times are not recommended.
6. Wash each sample with 200 µl **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 loaded with TFL4)
- **B.** Target cells + Substrate from Vial G_2D_2
- **C.** Effector cells
- **D.** Effector cells + Substrate from Vial GS
- **E.** Target cells + Effector cells + Substrate from Vial G_2D_2 (multiple samples).

Flow Cytometry: Please use settings consistent with the following excitation and emission peaks:

TFL4 λ_{ex}: 633 nm, λ_{em}: 657 nm

G_2D_2 λ_{ex}: 555 nm, λ_{em}: 580 nm

1. Use samples **A** and **D** to initially set the Red and Green/Yellow channels, resp.: place the peak for cells from sample **A** near 10^3 in the Red channel and the peak from sample **D** at *ca.* 10^1 in the Green/Yellow channel. Cells in sample B should then be at *ca.* 10^3 in the Red and 10^1 in the Green/Yellow channels. (Note: Healthy (>95% viable by Trypan Blue) 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.)
2. Run remaining samples. 5,000-10,000 Target cells per sample should be collected for analysis.

Selected References:

1. Visualization and quantification of T cell-mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. Liu *et al*. **Nature Med.** *8*:185-189 (2002).
2. Assessment of lymphocyte-mediated cytotoxicity using flow cytometry. Telford *et al*. **Methods Mol. Biol.** *263*:125-140 (2004).
3. Cutting Edge: Rapid In Vivo Killing by Memory CD8 T Cells. Barber *et al*. **J. Immunol.** *171*:27-31 (2003).
4. FcγRs Modulate Cytotoxicity of Anti-Fas Antibodies: Implications for Agonistic Antibody-Based Therapeutics. Xu *et al*. **J. Immunol.** *171*:562-8 (2003).
5. The Bcl-2 family pro-apoptotic molecule, BNIP3 regulates activation-induced cell death of effector cytotoxic T lymphocytes. Wan *et al*. **Immunology** *110*:10-7 (2003).
6. HIV-1-specific cytotoxicity is preferentially mediated by a subset of CD8(+) T cells producing both interferon-gamma and tumor necrosis factor-alpha. Lichterfeld *et al*. **Blood** *104*:487-94 (2004).
7. LT1-mediated T cell trafficking is critical for rejection and obliterative bronchiolitis after lung transplantation. Medoff *et al*. **J. Exp. Med.** *202*:97-110 (2005)
8. Cytotoxic herpes simplex type 2-specific, DQ0602-restricted CD4 T+-cell clones show alloreactivity to DQ0601. Reichstetter *et al*. **Immunology** *117*:350-7 (2005)
9. Respiratory syncytial virus G protein and G protein CX3C motif adversely affect CX3CR1+ T cell responses. Harcourt *et al*. **J. Immunol.** *176*:1600-8 (2006)
10. Stat1 deficiency in the host enhances interleukin-12-mediated tumor regression. Torrero *et al*. **Cancer Res.** *66*:4461-7 (2006).

Sample Flow Cytometry Data

Target cells (Jurkat, K562, or Daudi) were incubated in 75 µl Granzyme B Substrate with or without Effector cells (NK or CTL) at a 5:1 Effector:Target ratio for 60 minutes. (CTL assays were carried out using a redirected cytotoxicity protocol. Please contact OncoImmunin, Inc. for details.) Quadrants **R1** (upper left of each panel) represent viable target cells while quadrants **R2** (upper right) represent dying, Granzyme B-positive target cells. Effector cells occupy the lower 2 quadrants in Effector + Target samples. The inset % values are calculated as **R2**/(**R1**+**R2**).