OncoImmunin, Inc.

GranToxiLux®

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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 target cells following the successful delivery of a lethal hit by cytotoxic lymphocytes. 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.

GranToxiLux® is similar to CyToxiLux® PLUS! and PanToxiLux™, OncoImmunin, 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 and PanToxiLux™ detects *both* granzyme B and upstream caspase activities. As with the other two kits, GranToxiLux® 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 GranToxiLux®, CyToxiLux®, and PanToxiLux™ 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 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 GranToxiLux® kit (sufficient for 50 assays)

Vial GS (3 vials) = Granzyme B 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
TFL dilution medium (2 small Eppendorf tubes)=Resuspension medium for Vials TFL2 and TFL4
Wash Buffer bottle (1 bottle)

Components supplied by user

Effector cells
Target cells

In order to eliminate target cells that have died prior to addition of effector cells, e.g., due to a freeze/thaw cycle, please contact OncoImmunin, Inc. Please note: labeling of cell surfaces with fluorescent probes is **not** recommended as this may interfere with effector-target interactions.

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

Reconstitution of TFL2 or TFL4: Add 25 µl from one of the TFL dilution 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 below; rather, they are dyes with fluorescence properties more complementary to those of the Granzyme B Substrate.)

Medium T = Medium for labeling target cells. 1 µl from either reconstituted TFL2 or 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 TFL 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 TFL2 or 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.) 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 TFL2 or TFL4 in PBS for 15' at 37 °C is recommended. If sensitizers are to be used with PBLs, the recommendation is to add TFL2 or 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. Resuspend labeled Target cells at $1x10^6$ cells/ml in **Wash Buffer**. (Depending on the experimental design, lower numbers of Target cells may be used.)
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 **Wash Buffer**. For example, for a final Effector to Target ratio of 5:1, suspend Effector cells at $5x10^6$ 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 GS 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 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 for a given cell system should be significantly shorter than with other methodologies. For single point assays, the suggested coincubation time is 1 hour (enduser times range between 30 minutes and 2 hours). Substantially 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 TFL2 or TFL4)
- **B.** Target cells + Substrate from Vial GS
- **C.** Effector cells
- **D.** Effector cells + Substrate from Vial GS
- **E.** Target cells + Effector cells + Substrate from Vial GS (multiple samples)

Flow Cytometry: the following channels are recommended: APC for TFL4, PE for TFL2 and FITC for CG. For instruments with other filter sets, please use settings consistent with the following excitation and emission peaks:

TFL4 λ_{ex}: 633 nm, λ_{em}: 657 nm
TFL2 λ_{ex}: 488 nm, λ_{em}: 580 nm
GS λ_{ex}: 488 nm, λ_{em}: 520 nm

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 APC and FITC 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. (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.

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 APC and FITC channels, resp. Place the peak from sample **A** near 10^3 in the APC channel and and the peak from sample **D** at *ca.* 10^1 in the FITC channel. (Note: Healthy (>95% viable by Trypan Blue) TFL2-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. Use sample **B** to set up PE compensation. Dead/dying Effector cells may show a high FITC x PE population on most single-laser flow cytometers. Compensate PE by FITC until this population is on the same horizontal axis as viable Effector cells (low PE).
3. Run remaining samples. 5,000-10,000 Target cells per sample should be collected for analysis.
   (Note: channel amplification voltage settings for FITC and PE after these 3 steps should be similar in magnitude for ease in compensation. Should the PE voltage be greater than FITC by more than 100, then increase TFL2 labeling concentration or remove or reduce serum content.)

References for GranToxiLux®

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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 cytotoxocity 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**).