OncoImmunin, Inc.

NFL1

(Updated 05/06/09)

Overview

NFL1 is OncoImmunin, Inc.'s probe for eliminating target cells that have died prior to the start of experiments in which CyToxiLux®, GranToxiLux®, or PanToxiLux™ are used to detect cell-mediated cytotoxicity. Target cells are suspended in a solution containing NFL1 and TFL4, washed and then exposed to a cell permeable substrate with or without effectors. Cells that are positive for NFL1 are gated out; thus, the background of target cells due to low viability resulting from freeze/thaw cycles or suboptimal culturing is eliminated. Please note: both NFL1 and TFL4 are intracellular stains and, therefore, do not interfere with recognition between effectors and targets.

Please read this entire protocol before commencing assay!

NFL1 is supplied as a 1000X solution (for most applications) and should be stored at 4 °C.

Reconstitution of TFL4 (from CyToxiLux®, GranToxiLux®, or PanToxiLux™ kits)**:** 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 and 1 µl from the supplied NFL1 solution are 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 and NFL1 dilutions are both 1:1000 whereas for serum-free buffers, TFL4 is typically used at 1:3000 but NFL1 dilution may remain at 1:1000; however, further dilution, particularly of the TFL4 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.* A 1:1000 dilution of NFL1 can be used with most media.

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 and label as sample A. (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.) A second tube, labeled sample Ac, of approximately $1x10^6$ cells in 500 µl of Medium T minus NFL1 should also be incubated under the same conditions. 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 both TFL4 and NFL1 in PBS for 15' at 37 °C is recommended. If sensitizers are to be used with PBLs, the recommendation is to add TFL4 and NFL1 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 used.) Set aside sample Ac until flow cytometry measurements commence and continue setup with Target cells in sample A.
5. Dispense 100 µl of Target cell suspension (sample A) 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 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:

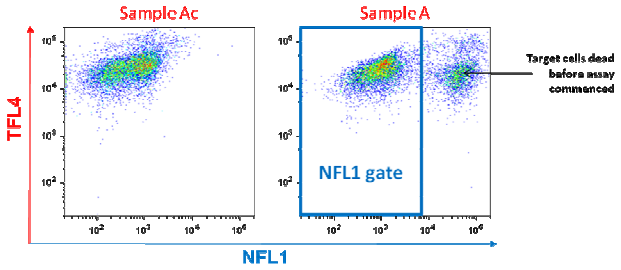
- **A.** Target cells
  - **A.** Target cells loaded with TFL4 and NFL1
  - **Ac.** Target cells loaded with TFL4 only.
- **B.** Target cells (sample A) + Substrate
- **C.** Effector cells
- **D.** Effector cells + Substrate
- **E.** Target cells(sample A) + Effector cells + Substrate (multiple samples)

Flow Cytometry: the following channels are recommended: PacBlue for NFL1, APC for TFL4, and FITC for substrate. For instruments with other filter sets, please use settings consistent with the following excitation and emission peaks:

NFL1 λ_{ex}: 405 nm, λ_{em}: 450 nm
TFL4 λ_{ex}: 633 nm, λ_{em}: 657 nm
GS λ_{ex}: 488 nm, λ_{em}: 520 nm

For flow cytometers equipped with Violet (405), Ar ion (488 nm) and red He-Ne (633 nm) lasers:

1. Use samples **A** and **Ac** to eliminate target cells that have died prior to experiment. Using the PacBlue and APC channels, apply an NFL1 gate to sample **A** as shown below and collect all cells contained therein.



2 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.)

3 Run remaining samples. 5,000-10,000 Target cells per sample should be collected for analysis.