Measuring Fluorescence Using the ApoTox-Glo™ Triplex Assay With the GloMax® Discover System

Promega Corporation

Materials Required

- ApoTox-Glo™ Triplex Assay (Cat.# G6320 and G6321)
- GloMax® Discover System (Cat.# GM3000)
- 96-well white plate

Caution: We recommend the use of gloves, lab coats and eye protection when working with these or any chemical reagents.

Protocols: *ApoTox-Glo™ Triplex Assay Technical Manual* #TM322 are available at: **www.promega.com/protocols/**

The ApoTox-Glo™ Triplex Assay combines three Promega assay chemistries to assess viability, cytotoxicity and caspase activation events within a single assay well. When used in combination with the GloMax® Discover System the pair provides a convenient, rapid and sensitive procedure, utilizing both fluorescent and luminescent quantitation, to determine specific cellular physiological conditions.

The first part of the assay simultaneously measures two protease activities; one is a marker of cell viability, and the other is a marker of cytotoxicity. Because live-cell protease activity is restricted to intact viable cells, a fluorogenic, cell-permeant peptide substrate (glycylphenylalanyl-aminofluorocoumarin; GF-AFC) can be used to generate a fluorescent signal proportional to the number of living cells. This live-cell protease becomes inactive upon loss of cell membrane integrity and leakage into the surrounding culture medium. A second, fluorogenic cell-impermeant peptide substrate (bis-alanylalanyl-phenylalanyl-rhodamine 110; bis-AAF-R110) is used to measure dead-cell protease activity, which is released from cells that have lost membrane integrity. These live- and dead-cell proteases produce different products, AFC and R110, which have different excitation and emission spectra, allowing them to be detected simultaneously.

The second part of the assay uses the Caspase-Glo® Assay Technology by providing a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and cell lysis. Adding the Caspase-Glo® 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal produced by luciferase. Luminescence is proportional to the amount of caspase activity present.

The ApoTox-Glo™ Triplex Assay is made easy on the GloMax® Discover System. The minimal well-to-well cross talk of the GloMax® Discover System allows the user to easily measure various sample signal intensities on the same plate. Fluorescence and luminescence readings are collected using the GloMax® Discover System (Figure 1). This Application Note describes the protocol for measuring fluorescence using the GloMax® Discover System with the ApoTox-Glo™ Triplex Assay.

Example Assay Protocol for 96-Well Plate Format

For detailed instructions and assay notes, see the *ApoTox-Glo™ Triplex Assay Technical Manual* #TM322. The following procedure can be used for measuring cell viability, cytotoxicity and apoptosis in cell-based assays in a 96-well plate format.

Reagent Preparation

1. Thaw each assay component as follows:
   - Assay Buffer: 37°C water bath
   - GF-AFC Substrate: 37°C water bath
   - bis-AAF-R110 Substrate: 37°C water bath
   - Caspase-Glo® 3/7 Buffer: room temperature
   - Caspase-Glo® 3/7 Substrate: room temperature
2. Transfer 10µl GF-AFC Substrate and 10µl bis-AAF-R110 Substrate into 2.0ml of Assay Buffer. Mix the Assay Buffer containing substrates by vortexing the contents until the substrates are thoroughly dissolved. This mixture will be referred to as the Viability/Cytotoxicity Reagent.
3. Transfer the contents of the Caspase-Glo® 3/7 Buffer bottle into the amber bottle containing Caspase-Glo® 3/7 Substrate. Mix by swirling or inverting the contents until the substrate is thoroughly dissolved to form the Caspase-Glo® 3/7 Reagent (~20 seconds).

   Note: See Technical Manual #TM322 for reagent storage information.

Protocol

1. Set up 96-well assay plates containing cells in medium at the selected density. We recommend using <20,000 cells per well in a 96-well plate.
2. Add test compounds and vehicle controls to appropriate wells for a final volume of 100µl per well.
3. Culture cells for the desired test exposure period. When characterizing new compounds, it is important to use in multiple exposure periods to assess the full effect on cellular health.
4. Add 20µl of Viability/Cytotoxicity Reagent containing both GF-AFC Substrate and bis-AAF-R110 Substrate to all wells, and briefly mix by orbital shaking (300–500rpm for ~30 seconds).
5. Incubate for 30 minutes at 37°C. Incubations longer than 30 minutes may improve assay sensitivity and dynamic range. However, do not incubate more than 3 hours.
6. Measure fluorescence on the GloMax® Discover System at the following two wavelength sets:
   - $405_{Ex}/495–505_{Em}$ (Viability)
   - $475_{Ex}/500–550_{Em}$ (Cytotoxicity)
7. Add 100µl of Caspase-Glo® 3/7 Reagent to all wells, and briefly mix by orbital shaking (300–500rpm for ~30 seconds). Incubation times longer than 30 minutes may improve assay sensitivity and dynamic range. See Note in the Before You Begin Section of Technical Manual #TM322 for more information.
8. Incubate for 30 minutes at room temperature.
9. Measure luminescence on the GloMax® Discover System.

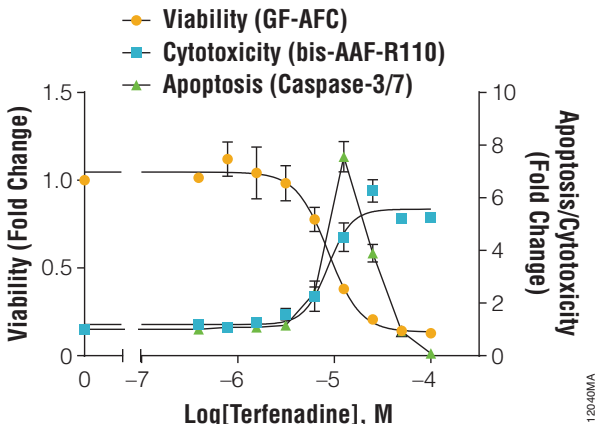

Figure 1. Expected results for terfenadine treatment of HepG2 cells. Terfenadine treatment for 3 hours should result in a dose-dependent decrease in viability, increase in cytotoxicity and caspase-3/7 activity, which is consistent with apoptosis.

GloMax® Discover System

The GloMax® Discover System offers the ability to measure distinct and varied wavelengths with limited well-to-well cross talk. The instrument was developed and optimized with Promega cell and gene reporter assays and may be integrated into low- and medium-throughput automation workflows. The GloMax® Discover System also provides flexible use of filters for fluorescence intensity, filtered luminescence, BRET, FRET and UV-visible absorbance measurements for adaptation into a wide variety of laboratory applications. The instrument is operated by an integrated tablet PC, which provides quick and easy navigation through the control options. Exporting your results is made seamless with a variety of options, including exporting data to your local network. Thus together with the ApoTox-Glo™ Triplex Assay, the GloMax® Discover offers a complete and easy to use solution to measure viability, cytotoxicity and caspase activity of cells.

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