Determining LDH Cytotoxicity in 3D Microtissues with the GloMax® Discover System

Promega Corporation

Materials Required

- LDH-Glo™ Cytotoxicity Assay (Cat.# J2380)
- GloMax® Discover System (Cat.# GM3000)
- Corning® Costar® Ultra-Low Attachment Multi Well Plates, black/clear bottom (Corning® Cat.# 4515)
- White, 96-well assay plates (Corning® Cat.# 3917)
- LDH Storage Buffer
- Optional: CellTiter-Glo® 3D Cell Viability Assay (Cat.# G9681)

Caution: We recommend the use of gloves, lab coats and eye protection when working with these or any chemical reagents.
Protocols: *GloMax® Discover System Technical Manual* #TM397 and *LDH-Glo™ Assay Technical Manual* #TM548 are available at:
www.promega.com/protocols

Overview

Lactate dehydrogenase (LDH) is a stable, soluble cytosolic enzyme present in many cell types. LDH is rapidly released into the cell culture medium upon disruption of the plasma membrane, and thus it is widely used as a marker of cytotoxicity. Increasing concentrations of LDH can be detected in culture medium as cytotoxicity progresses.

The LDH-Glo™ Cytotoxicity Assay is a sensitive, plate-based, bioluminescent method for measuring LDH release. With other colorimetric or fluorescence LDH detection methods, it can be difficult to determine cytotoxicity in samples with low cell number, such as 3D microtissue spheroids, microfluidic cell culture chips, primary cells and stem cells. The bright luminescent signal provided by the LDH-Glo™ Cytotoxicity Assay is an ideal system for such sample types. As LDH is released into the cell culture medium, it catalyzes the oxidation of lactate with the reduction of NAD^+ to NADH. NADH is then used to facilitate reduction of luciferin, which becomes a substrate for luciferase-generated luminescence signal. The luminescence signal increases proportionally as the amount of LDH increases in the sample. The assay is performed by removing a small volume (i.e. 2–5µl) of the culture medium to assay. The remaining cells and media can then be used for additional assays, such as nucleic acid analysis or other cell health assays.

Measuring luminescence from the LDH-Glo™ Cytotoxicity Assay is easy on the GloMax® Discover System because the protocol comes preloaded on the instrument. The superior sensitivity, extended dynamic range and minimal well-to-well crosstalk of the GloMax® Discover System allow you to easily measure samples with low cell numbers. This Application Note describes a protocol to measure LDH release using the LDH-Glo™ Cytotoxicity Assay on the GloMax® Discover System. We also describe the measurement of cell viability using the remaining cells and the CellTiter-Glo® 3D Cell Viability Assay, a homogeneous, luminescent, plate-based method to determine cell viability based on the quantitation of ATP present in metabolically active cells.

LDH-Glo™ Cytotoxicity Assay Protocol

For detailed instructions and assay notes for various assay volumes and plate formats, see the *LDH-Glo™ Cytotoxicity Assay Technical Manual* #TM548. The following protocol is performed in 96-well plates.

1. Add test compound or vehicle control to prepared spheroids. Incubate for desired time at desired conditions.
2. Remove small amounts of media (2–5µl) from each treated spheroid well at desired timepoints. Take care to not touch or disturb the spheroids.
3. Dilute media in LDH Storage Buffer (1:20). If assay will be delayed, freeze the diluted media samples at –20°C.
4. On the day of assay, thaw the collected samples and further dilute in LDH Storage Buffer to be within linear range of the assay (e.g. 1:100).
5. Prepare the LDH Detection Reagent as specified in the technical manual. Ensure the Reductase Substrate is placed on ice before detection reagent preparation.
6. Add 50µl of prepared reagent to 50µl of sample in an opaque white 96-well plate.
7. Incubate for 60 minutes at room temperature.
8. Measure luminescence using a 0.3 second integration time on the GloMax® Discover by selecting the "LDH-Glo" protocol.

(Optional) CellTiter-Glo® 3D Cell Viability Protocol

For detailed instructions and assay notes for various assay volumes and plate formats, see the *CellTiter-Glo® 3D Cell Viability Assay Technical Manual* #TM412. The following protocol is performed in 96-well plates.

1. Prepare CellTiter-Glo® 3D Reagent as specified in the technical manual. Ensure reagent and cells are equilibrated to room temperature.
2. Add a volume of CellTiter-Glo® 3D Reagent equal to the volume of the cell culture medium remaining in each well (subtract the total volume of media removed for LDH measurement from the starting volume of media).
3. Place lid on plate and shake (700rpm) at room temperature for 30 minutes (you may need to adjust shaking/incubation time based on cell culture system used).
4. Measure luminescence using a 0.3 second integration time on the GloMax® Discover by selecting the "CellTiter-Glo" protocol.

Conclusion

The GloMax® Discover can detect and analyze luminescence generated using the LDH-Glo™ Cytotoxicity Assay and CellTiter-Glo® 3D Cell Viability Assay as shown in Figure 1. With increasing doses of Bortezomib to induce cytotoxicity and LDH release, increased luminescence was observed as expected. Different dose response curves were observed based on the time the cells were exposed to the drug, as expected. Even with samples containing low cell number, such as 3D spheroids, the GloMax® Discover was able to collect measurable signals.

The GloMax® Discover System

The GloMax® Discover System offers superior sensitivity, dynamic range and 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 allows flexible use of filters to measure fluorescence intensity, filtered luminescence, BRET, FRET and UV-visible absorbance for 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.

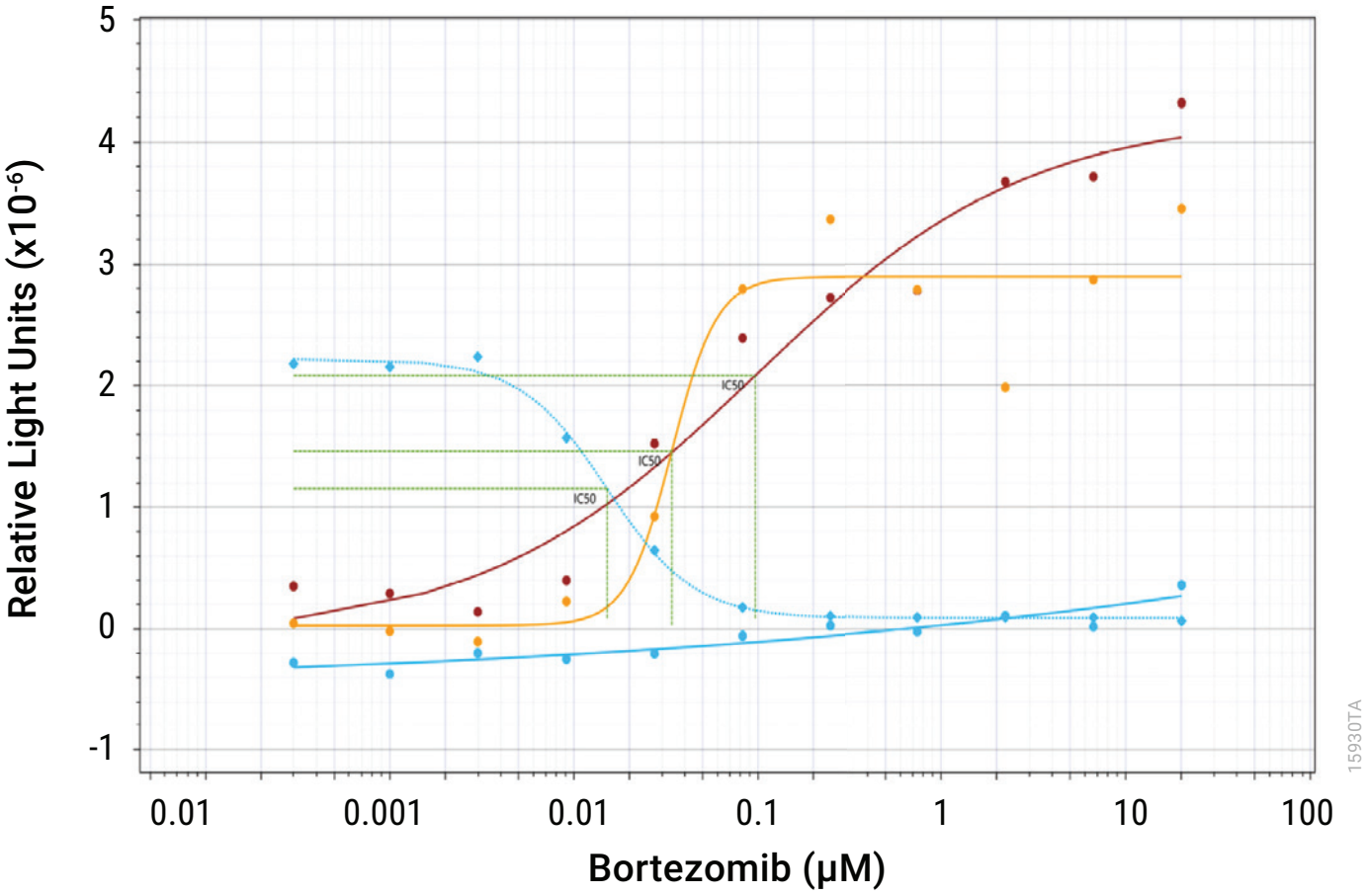

Figure 1. Dose-response curves generated using the LDH-Glo™ Cytotoxicity Assay from 3D (spheroid) human colorectal carcinoma microtissues (HTC116) multiplexed with CellTiter-Glo® 3D Viability Assay. HCT116 spheroids were grown in Ultra-Low Attachment Plates for 3 days at 37°C, 5% CO_2 and subsequently treated with Bortezomib (37°C, 5% CO_2) to induce LDH release. Media samples were taken from the spheroid plate at 24hrs, 48hrs and 64hrs and diluted with LDH Storage Buffer [200mM Tris-HCl (pH 7.3), 10% Glycerol, 1% BSA]. The diluted samples were assayed with the LDH-Glo™ Cytotoxicity Assay. Spheroids were then assayed with the CellTiter-Glo® 3D Viability Assay. Both plates were read on a GloMax® Discover System. The dose response curves were generated using the GloMax® Analysis Software. The image above displays the resulting dose response curves. The LDH curves are represented with circles: blue at 24hrs, red at 48hrs and yellow at 64hrs. The CellTiter-Glo® curve is represented with blue diamonds and a dotted line as a single endpoint curve.

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