

CYTOTox-ONE™ HOMOGENEOUS MEMBRANE INTEGRITY ASSAY: A TOOL FOR AUTOMATED CYTOTOXICITY RESEARCH

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This article describes the use of the CytoTox-ONE™ Homogeneous Membrane Integrity Assay on automated robotic platforms. We demonstrate automation effectiveness using cell lysis experiments, dose response curves, and Z' factor analysis.

Introduction

Robotic integration of cell-based assays for in vitro toxicity screening of chemical compounds is becoming an increasingly important aspect of the drug discovery process. Development of assays that simplify the automation process is crucial for the success of this high-throughput screening. Promega has recently introduced the CytoTox-ONE™ Homogeneous Membrane Integrity Assay^(a), a fluorometric method for estimating the number of nonviable cells present in multiwell plates. For a detailed description of the assay, see Riss and Moravec this issue.

Methods

Cell Lysis Experiments

HepG2 cells were plated in black plates with clear bottoms (Corning #3603), grown until confluent and treated with a serial dilution of CytoTox-ONE™ Lysis Solution. The Lysis Solution is supplied with the CytoTox-ONE™ Assay for use as a positive control for maximal LDH release. In these experiments, we also used it to mimic a drug dose response curve, allowing quick and easy robotic assay development.

Before beginning the assay, culture plates and CytoTox-ONE™ Assay Buffer were allowed to equilibrate to room temperature. To prepare the CytoTox-ONE™ Reagent, 11ml of Assay Buffer was added to the lyophilized Substrate Mix. To perform the CytoTox-ONE™ Assay, a two-plate protocol was written for the Beckman Biomek® 2000 Laboratory Automation Workstation. The robot added 100µl of CytoTox-ONE™ Reagent to each well followed by a short (approximately 30 seconds) mix on the integrated shaker. Plates were allowed to incubate for 10 minutes, after which the robot added 50µl of CytoTox-ONE™ Stop Solution. To distribute the Stop Solution evenly, the plates were again briefly shaken on the integrated shaker. For all experiments, plates were immediately read on a BMG POLARStar fluorescent plate reader equipped with a 544nm excitation and 620nm emission filter set.

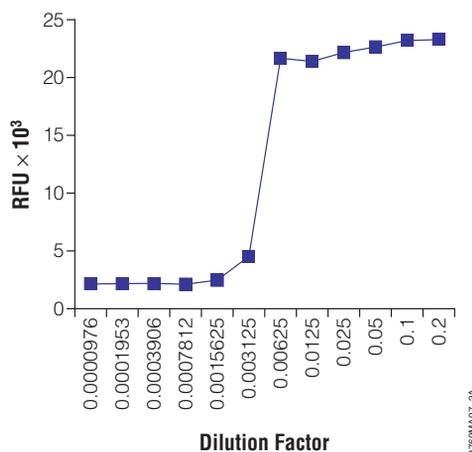


Figure 1. Lysis Solution dose response from a dilution factor of 0.2 (1:5 dilution) to a dilution factor of 9.76×10^{-5} (1:10,240 dilution) on a confluent layer of HepG2 cells in 96-well plates.

Staurosporine Dose Response

HepG2 human liver cells were plated in 96-well tissue culture, treated white plates with a clear bottom (Corning #3610) at a density of 40,000 cells/well. Cells were allowed to grow to confluence and subsequently treated for 24 hours with a 48nM to 25µM range of staurosporine in DMSO or the appropriate concentration of DMSO alone. The same Biomek® 2000 workstation protocol was used as in the lysis experiments described above.

Z' Factor Analysis

HepG2 cells were plated as above, allowed to become confluent and then treated with either 3.125µM staurosporine on one half of the plate or the DMSO vehicle control on the other half. The same Biomek® 2000 workstation protocol was used as in the dose response experiments described above.

Results and Discussion

Cell Lysis Experiments

Using the Cell Lysis Solution supplied with the CytoTox-ONE™ Assay allowed us to quickly and easily design and implement the Biomek® 2000 protocol without having to wait for the necessary drug treatment effect. The data also demonstrate the highly reproducible nature of both the assay itself and the automated method with %CVs below 5% for most "drug" concentrations. Figure 1 shows the Lysis Solution dose response from a dilution factor of 0.2 to 9.76×10^{-5} . Note the large drop off in RFU signal at a dilution factor of 3.125×10^{-3} . The

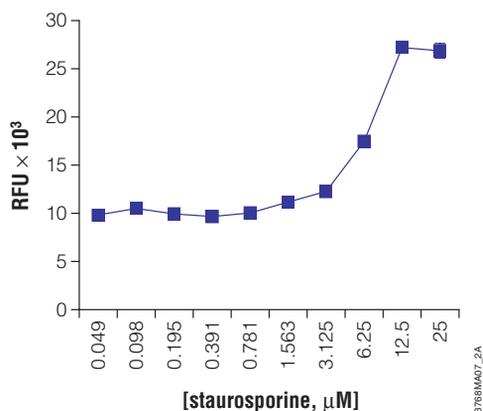


Figure 2. Staurosporine dose response curve. Confluent HepG2 cells were treated for 24 hours with staurosporine from 48nM to 25μM to generate a dose response curve. %CVs were below 5% for all drug concentrations showing the robustness of both the CytoTox-ONE™ Assay and the robotic platform.

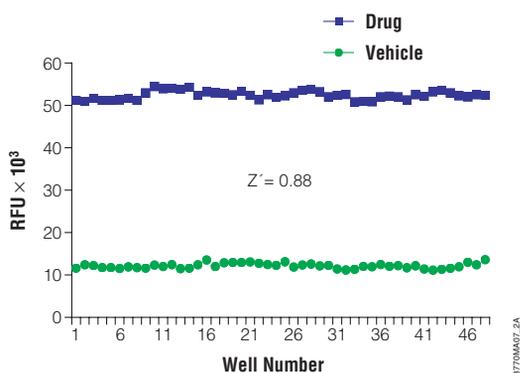


Figure 3. Representation of Z' factor equal to 0.88 for one of the plates processed by the Biomek® 2000 workstation. A scattergram was created for a 96-well plate of confluent cells; one half was treated with 3.125μM staurosporine and the other half with vehicle.

recommended amount of Lysis Solution for the positive control is 2μl per 100μl of culture medium, which would represent a dilution factor of 0.02 in Figure 1.

Staurosporine Dose Response

Figure 2 shows a typical staurosporine dose response curve from 48nM to 25μM. %CVs were below 5% for all drug concentrations showing robustness of both the CytoTox-ONE™ Assay and the robotic platform.

Z' Factor Determination

To determine assay robustness and reproducibility of the system, the statistical calculation for Z' factor was used (1). This "screening window coefficient" compares the assay dynamic range to data variation, making it a powerful tool to assess assay quality. Z' factor for each plate processed by the Biomek® 2000 workstation was calculated using the following formula:

$$1 - \frac{(3*SD \text{ of sample} + 3*SD \text{ of control})}{|(\text{mean of sample} - \text{mean of control})|}$$

A Z' factor equal to 1.0 is a perfect assay. Z' factors greater than or equal to 0.5 indicate an excellent assay. Figure 3 shows a representation of Z' factor equal to 0.88 for one of the plates processed by the Biomek® 2000 workstation. This indicates that the system used (the combination of Biomek® 2000 workstation processing and Promega's CytoTox-ONE™ Assay) provides an excellent assay.

Conclusion

Here we demonstrate integration of the CytoTox-ONE™ Assay on a medium-throughput robotic workstation, Beckman Coulter's Biomek® 2000 Laboratory Automation Workstation. Even though manual processing data for the CytoTox-ONE™ (see Riss and Moravec this issue) shows the benefit of a homogeneous assay, robotic integration lends itself to greater throughput and reproducibility over the long term. We have created a two-plate method on the Biomek® 2000 with room left for higher throughput. For users requiring greater throughput capabilities, the CytoTox-ONE™ Assay protocol was also implemented on the Beckman Coulter Biomek® FX Laboratory Workstation (data not shown).

Reference

- Zhang, J. *et al.* (1999) *J. Biomol. Screening* **4**, 67–73.

Protocol

CytoTox-ONE™ Homogeneous Membrane Integrity Assay Technical Bulletin #TB306

www.promega.com/tbs/tb306/tb306.html

Ordering Information

Product	Size	Cat.#
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	200–800 assays	G7890
	1,000–4,000 assays	G7891

(a)Patent Pending.
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