Bringing Automation To The Cytotoxicity Researcher— An ADME/Tox Application

Tracy Worzella, B.S.[1], Randy Hoffman, B.S.[1], Monika Walterschied, M.S.[2], Norman L. Sussman, M.D.[2]

Promega Corporation[1] and Amphioxus Cell Technologies[2]

Abstract

We describe the use of the CytoTox 96® Non-Radioactive Cytotoxicity Assay in a medium-throughput research application using the Biomek® 2000 Laboratory Workstation and the ACTIVTox® hepatocyte cell line (Figure 1).

Introduction

High-Throughput Screening (HTS) has become a phrase closely associated with cell-based assays. Until recently, pharmaceutical and biotech companies were the primary users of high-throughput, cell-based assays in their search for drugs that are effective, safe and profitable. Cells are plated in high-density culture plates, treated with drug libraries consisting of tens of thousands of candidates, and assayed for a particular effect. The entire process, from cell culture to data analysis, can be automated to allow for ultra high-throughput screening, keeping reproducibility high and cost per assay low.

However, a variety of lower-throughput automated platforms are available for use in the research laboratory. Each platform has a certain throughput associated with it, and the number of samples handled within a given time frame indicates the most appropriate platform. Figure 2 depicts several of the robotic platforms available for low-, medium- and high-throughput research applications. For more information regarding HTS considerations, please see the issue of *Drug Discovery and Development* dedicated to this topic (1).

This article demonstrates the value of automation for the researcher with low- to medium-throughput needs.

This article demonstrates the value of automation for the researcher with low- to medium-throughput needs. Automation has become more readily adaptable to these needs because of the recent availability of lower-cost, automation-amenable, cell-based assays along with the development of campus-wide or interdepartmental robotic core facilities. Here we provide an example of a medium-throughput toxicity assay system using Beckman Coulter's Biomek® 2000 laboratory workstation with

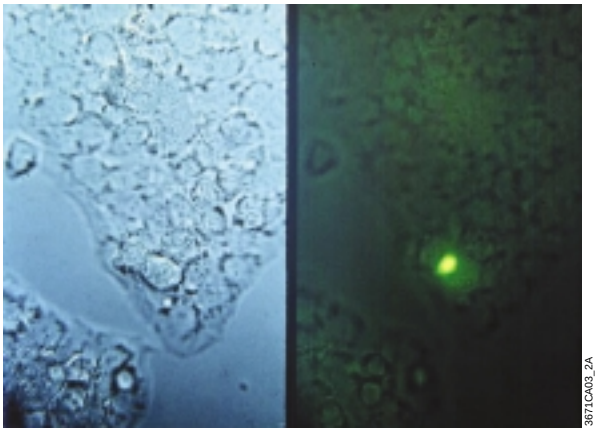

Figure 1. Image of Amphioxus Cell Technologies' ACTIVTox® cell line. Cultured cells viewed by light microscopy and fluorescence microscopy. The bright spot in the right hand panel is a bile canaliculus. Image reprinted with the kind permission of Norman L. Sussman, Amphioxus Cell Technologies (info@amphioxus.com).

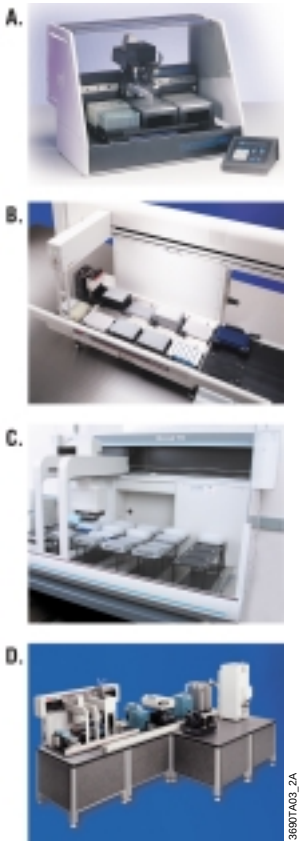

Figure 2. Automated platforms. The following automated platforms are examples of different systems with different throughput capabilities. The platforms are shown from lower-throughput to higher-throughput. Platform B was used in this article.
Panel A. BioTek Precision 2000™ (low throughput).
Panel B. Beckman Biomek® 2000 (medium throughput).
Panel C. Biomek® F/X (high throughput).
Panel D. SAGIAN™ Core System (high throughput).

Promega's CytoTox 96® Non-Radioactive Cytotoxicity Assay and Amphioxus Cell Technologies' ACTIVTox® cell line (Figure 1). This system allows for reproducible and quick assay setup with the capability of processing up to three 96-well culture plates at a time. In this article, a two-plate method was written and used. Culturing, drug treatment and plate reading are done offline, adding to the flexibility of the system.

The ACTIVTox® cells used in this study are a patented and highly selected subclone of HepG2 that have retained many of the properties of normal adult hepatocytes such as glucogenesis, albumin production, and drug metabolism. This "adult" phenotype makes these cells a potential model for ADME/Tox (Absorption, Distribution, Metabolism, Elimination/Toxicity) screening in a high-throughput format.

Promega's CytoTox 96® Non-Radioactive Cytotoxicity Assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a coupled enzymatic assay that results in the conversion of a tetrazolium salt into a red formazan product. The amount of color formed is proportional to the number of cells lysed.

To determine assay robustness and reproducibility of the system, we have used the statistical calculation for Z´-factor (2). This "screening window coefficient" compares the assay dynamic range to data variation, making it a powerful tool to assess assay quality.

Materials and Methods

Several 96-well plates seeded with ACTIVTox® cells (Amphioxus Cell Technologies; info@amphioxus.com; 281-679-7900) were allowed to mature for one week at Amphioxus Cell Technologies. The cells, fresh medium and new sterile lids were then shipped at ambient temperature to Promega Corporation. Upon receipt of the cells, the culture medium was replaced with 200µl of fresh medium. The plates were covered with a new sterile lid and incubated overnight in a 37°C, 5% CO_2 incubator to allow the cells to recover from the shipment.

After the overnight recovery period, we treated the cells with a serial dilution of staurosporine to determine effective dose range. Appropriate control wells were treated with the corresponding concentration of DMSO vehicle. Cells were treated for 48 hours at 37°C, 5% CO_2 followed by assay setup on the Biomek® 2000 with Promega's CytoTox 96® Non-Radioactive Cytotoxicity Assay (Figure 3, Table 1).

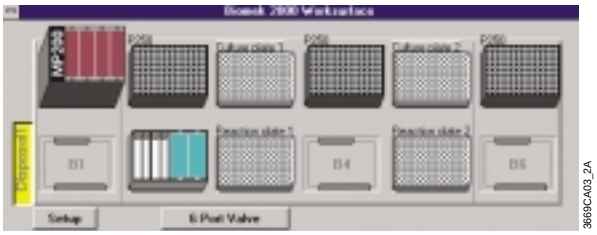

Figure 3. Deck configuration of the Biomek® 2000 for the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Cat.# G1780). The only tool needed is the MP200 tool positioned at A1. Positions B1, B4 and B6 remain empty. P250 tip boxes are placed at positions A2, A4 and A6. Position B2 holds a reservoir that contains 8ml of Nuclease-Free Water (Cat.# P1193), 12ml of CytoTox 96® Substrate and 12ml of CytoTox 96® Stop Solution. The 96-well culture plates containing the ACTIVTox® cells are placed at positions A3 and A5. Two empty, flat-bottom 96-well plates are placed at positions B3 and B5.

Table 1. Biomek® 2000 Program for Performing the CytoTox 96® Non-Radioactive Cytotoxicity Assay with ACTIVTox® Cells.

1. The Biomek® 2000 transfers 25µl of nuclease-free water from the reagent reservoir to the reaction plates.
2. The Biomek® 2000 transfers 50µl of CytoTox 96® Substrate from the reagent reservoir to the reaction plates.
3. 25µl of the culture medium is transferred from the culture plate to the reaction plates.
4. The Biomek® 2000 pauses and prompts the user to cover the 96-well flat bottom plates with foil. The plates remain on the deck for a 15 minute room temperature incubation. After the 15 minute incubation, the user removes the foil from the plates and cancels the system pause in order for the Biomek® to continue with the assay.
5. 50µl of the CytoTox 96® Stop Solution is transferred from the reagent reservoir to the reaction plates.
6. The user removes the reaction plates from the deck of the Biomek® 2000 and reads A_{490} on a plate reader.

Figure 3 illustrates the deck layout and position of the 96-well plate of ACTIVTox® cells and other components required for a two-plate assay. Table 1 outlines the program for performing the CytoTox 96® Non-Radioactive Cytotoxicity Assay. The entire Biomek® 2000 protocol, which includes a 15-minute incubation, takes approximately 25 minutes to complete. After method completion, the reaction plates were removed from the deck of the Biomek® 2000, and absorbance readings at 490nm were recorded using a Molecular Devices SpectraMAX® plate reader.

Table 2. Statistics Used to Calculate Z′-Factor for Each Plate Processed by the Biomek® 2000.

	Staurosporine Treated			Vehicle Control			Z′-factor
	Mean A_{490}	SD	%CV	Mean A_{490}	SD	%CV	
Plate 1	3.996	0.090	2.26	0.602	0.064	10.57	0.864
Plate 2	3.536	0.195	5.52	0.461	0.030	6.57	0.780
Plate 3	3.596	0.099	2.74	0.659	0.066	10.07	0.831
Plate 4	3.134	0.140	4.47	0.498	0.056	11.12	0.778

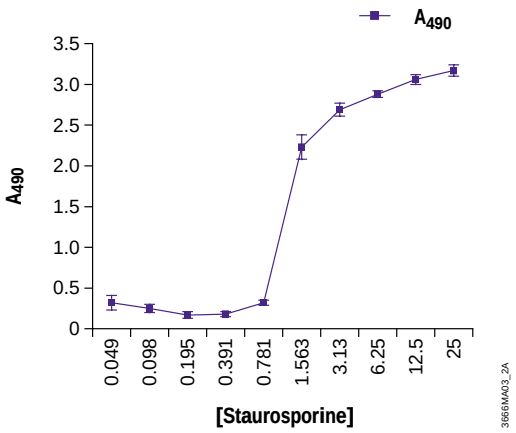

Figure 4. Dose response curve for staurosporine. The CytoTox 96® Non-Radioactive Cytotoxicity Assay was used to determine the effective dose for staurosporine.

The dose response curve shows that 3.13µM is an appropriate cytotoxic concentration of staurosporine (Figure 4). For Z′-factor determination, we treated one-half of a 96-well plate of ACTIVTox® cells with 3.13µM staurosporine and the other half with the DMSO vehicle control. The cells were then incubated in the presence of drug and vehicle for 48 hours in a 37°C, 5% CO_2 incubator.

Results

Z′-factor for each plate processed by the Biomek® 2000 was calculated using the following formula:

$$Z' = 1 - \frac{(3\ \text{S.D. pos.control} + 3\ \text{S.D. neg. control})}{\left|(\text{mean pos. control} - \text{mean neg. control})\right|}$$

A Z′-factor equal to 1.0 is a perfect assay. Z′-factors greater than or equal to 0.5 indicate an excellent assay. All four plates had Z′-factors greater than 0.5 (Table 2), indicating that the system provides for a robust assay.

Conclusion

Here we show that medium-throughput cytotoxicity assays are within the reach of laboratory researchers. The combination of a common robotic workstation such as the Biomek® 2000 with the CytoTox 96® Non-Radioactive Cytotoxicity Assay and the ACTIVTox® cell line provides researchers with an integrated cytotoxicity solution. The ACTIVTox® cells circumvent many of the prevailing issues inherent in using human adult primary liver cells or nonhuman liver cell lines. By using Z′-factor as an indicator of assay robustness, we have shown that the combination of these three components can satisfy the needs of the medium-throughput cytotoxicity researcher.

References

1. *Drug Discovery & Development* (2002) **5**, entire issue.
2. Zhang, J. *et al*. (1999) *J. Biomol Screening* **4**, 67–73.

Protocols

CytoTox 96® Non-Radioactive Cytotoxicity Assay Technical Bulletin #TB163, Promega Corporation
www.promega.com/tbs/tb163/tb163.html

Ordering Information

Product	Size	Cat.#
CytoTox 96® Non-Radioactive Cytotoxicity Assay	1,000 assays	G1780

For Laboratory Use.