HIGH-THROUGHPUT AUTOMATION OF MULTIPLEXED CELL-BASED ASSAYS FOR VIABILITY AND CYTOTOXICITY

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Introduction

Modern drug discovery programs are increasingly dependent upon cell-based assays as a means of screening compound libraries (1). Such an approach allows the interrogation of specific agonistic or antagonistic signaling events within a cellular context while also assessing issues of compound permeability and stability. More importantly, cell-based assays allow the researcher to address potential global effects resulting from cytostatic or cytotoxic events that may affect the validity of specific assay measures. We have developed reagents that allow same-well, simultaneous or sequential measurement of both viability and cytotoxicity. These assays are fully compatible with a battery of other spectrally distinct third-parameter assays. Here we demonstrate how the assays perform on the CyBio CyBi®-Well automation platform in high-throughput and ultrahigh-throughput cell-based multiplexing studies.

Assay Principles

The MultiTox Assays (Figure 1) use differential protease biomarker detection to quantify the number of live and dead cells simultaneously in a single well (2). These assays allow you to normalize for well-to-well variation that can be introduced when cells are plated, test compounds are added, or reagents are dispensed (1–4).

Automation Provides Robust Results

The MultiTox Assays are designed to be scaleable while retaining their sensitivity, even in miniaturized formats. In this article we will highlight the ease of use of these assays in 384- and 1536-well assay formats.

We used the CyBio CyBi®-Well 384/1536 pipettor (Figure 2) for dispensing cells, adding treatment compounds and adding assay reagents. In these studies, we used a four-position plate carrier and 25 µl disposable tip cartridges for the different dispensing steps.

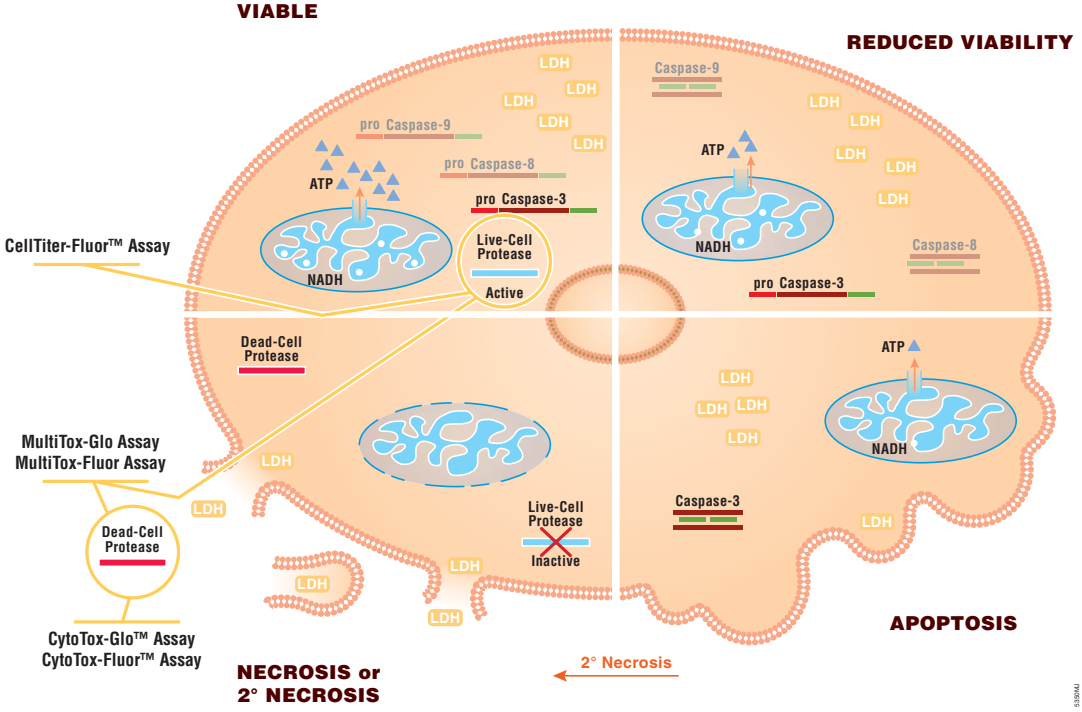

Figure 1. The assay principle behind the MultiTox-Fluor, MultiTox-Glo, CytoTox-Glo™, CytoTox-Fluor™ and CellTiter-Fluor™ Assays. The live-cell protease activity is measured by the fluorogenic, cell-permeant peptide substrate Gly-Phe-7-amino-4-trifluoromethyl coumarin (GF-AFC). This live-cell protease activity marker becomes inactive upon loss of membrane integrity and leakage into the surrounding culture medium. The second, dead-cell protease activity marker is measured from cells that have lost membrane integrity. In the MultiTox-Fluor Multiplex Cytotoxicity Assay, this activity is measured with the cell-impermeant peptide substrate, bis-(Ala-Ala-Phe)-rhodamine 110 (bis-AAF-R110). The MultiTox-Glo Multiplex Cytotoxicity Assay measures this dead-cell protease activity with the cell-impermeant luminogenic substrate, Ala-Ala-Phe-aminoluciferin (AAF-Glo™ Substrate).

Figure 2. The CyBi®-Well Instrument is a high-precision contact dispenser with simultaneous pipetting capabilities. The instrument also includes a stationary pipetting head (lower picture) with 384 channels, a plate lifter for moving plates up to the tips and a stage for indexing plates to access quadrants in a 1536-well plate. The CyBi®-Well Instrument has a modular design that enables its use as a bench-top pipettor for assay development (for example in addition with stackers or a CyBi® Drop), or it can be incorporated into larger screening systems for higher throughput.

Compatibility with Downstream Assays

The nonlytic formulation of the MultiTox Assays allows you to perform them in multiplex with other downstream assays. Cells can be interrogated for additional information such as apoptosis signaling or reporter gene expression.

We performed each MultiTox Assay in multiplex with a compatible apoptosis assay in a 384-well and 1536-well format: the luminescent Caspase-Glo® 3/7 Assay was performed following the MultiTox-Fluor Assay (Figure 3), and the fluorescent Apo-ONE® Homogeneous Caspase-3/7 Assay was performed following the MultiTox-Glo Assay (Figure 4). The data in Figure 5 show that, with increasing concentration of treatment compound, there is a decrease in cell viability and a corresponding increase in cell death. The apoptosis assays confirm that caspase-3/7 activity increases over the dose range, suggesting that cells are undergoing apoptosis. The data in Figure 6 are from a 1536-well format and demonstrate the high-quality of the assays as indicated by Z′-factor analysis.

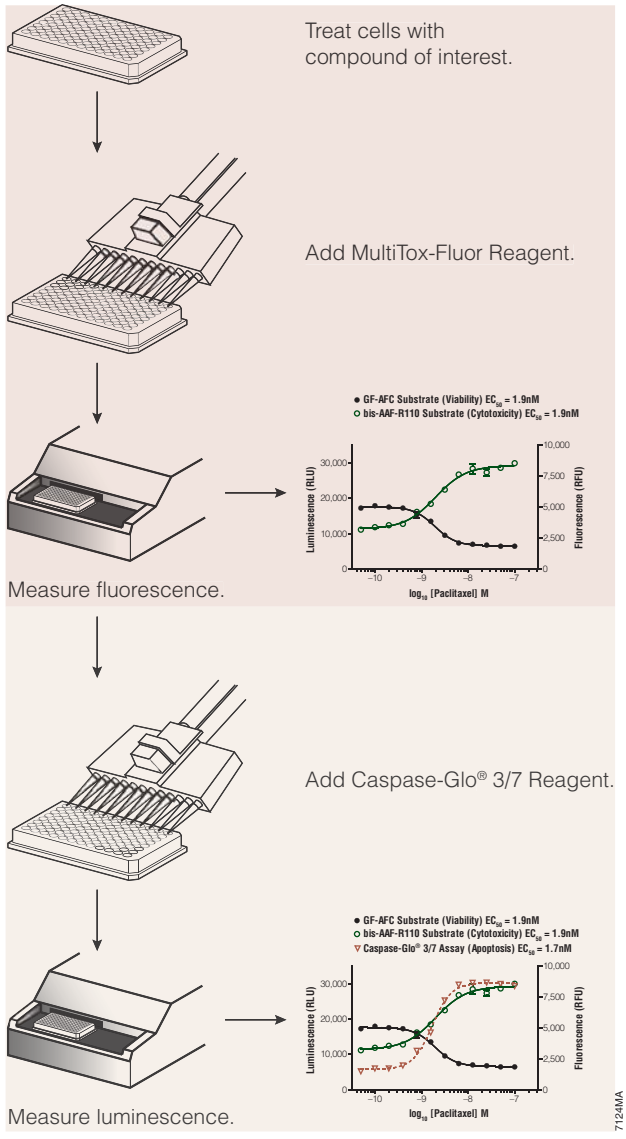

Figure 3. Flow chart outlining the required steps for high-throughput MultiTox-Fluor Assays performed alone and in multiplex with the Caspase-Glo® 3/7 Assay. Darker shaded box highlights the MultiTox-Fluor Assay steps; the lighter box indicates the Caspase-Glo® Assay steps. Reagents were prepared and added as described in Table 1.

Table 1. Assay Volumes for Standard and Multiplex Protocols for MultiTox-Fluor and Caspase-Glo® Assays.

Cells/Reagent	Standard Protocol 384-well (1536-well)	Multiplex Protocol with Caspase-Glo Assay 384-well
Cells and Treatment Compound	20 µl (4 µl)	20 µl
MultiTox-Fluor Reagent*	20 µl (4 µl)	5 µl
Caspase-Glo® Reagent	None	25 µl

*For standard protocol, the MultiTox-Fluor reagent was prepared by adding 10 µl of each substrate to 10 ml of assay buffer. For the multiplex protocol, the reagent was prepared by adding 10 µl of each substrate to 2.5 ml of assay buffer.

Multiplex Cytotoxicity Assays

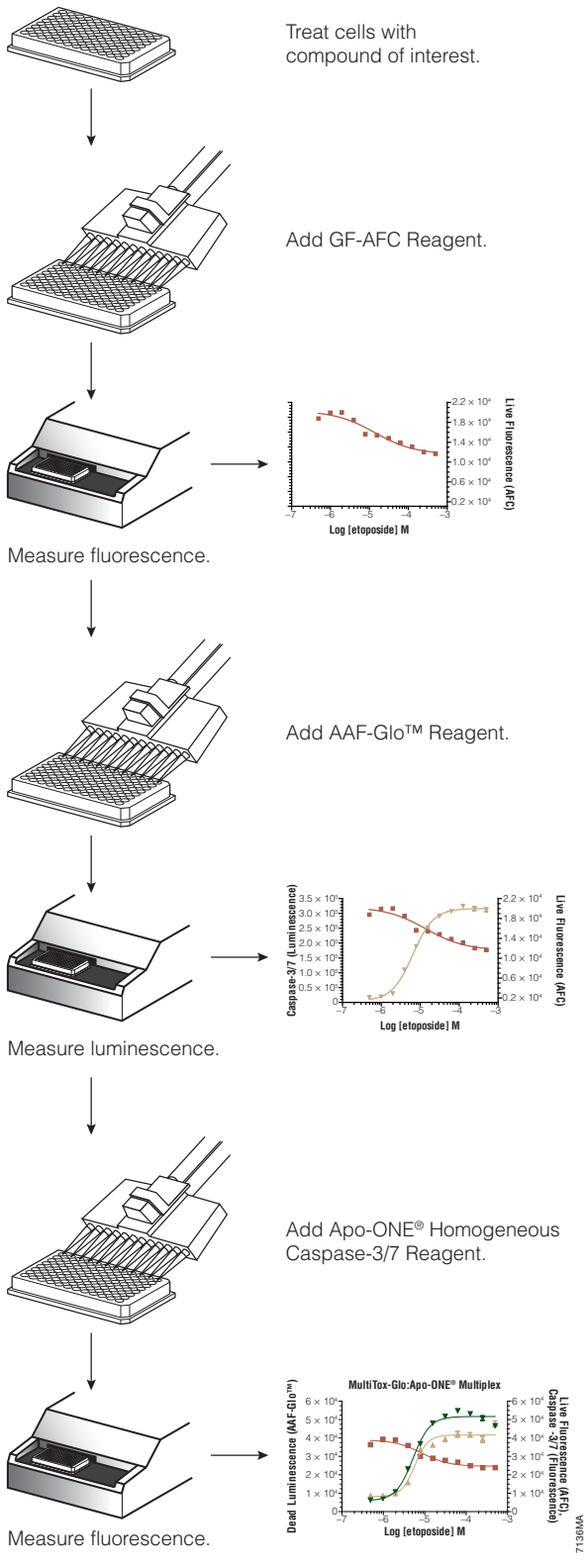

Figure 4. Schematic outlining the protocol for a standard MultiTox-Glo Assay (tan box) and a multiplexed Apo-ONE® Assay (green box). Volumes and preparation of reagents are described in Table 2.

Table 2. Assay Volumes for Standard and Multiplex Protocols for MultiTox-Glo and Apo-ONE® Assays.

Cells/Reagent	Standard Protocol 384-well (1536-well)	Multiplex Protocol with Apo-ONE® Assay 384-well
Cells and Treatment Compound	20 µl (4 µl)	20 µl
GF-AFC Reagent*	10 µl (2 µl)	10 µl
AAF-Glo™ Reagent*	10 µl (2 µl)	10 µl
Apo-ONE® Reagent	None	40 µl

*For standard protocol, each reagent was prepared by adding 10 µl of substrate to 10 ml of assay buffer. For the multiplex protocol, each reagent was prepared by adding 10 µl of substrate to 2.5 ml of assay buffer.

Summary

The MultiTox-Fluor and MultiTox-Glo Assays allow you to measure live and dead cells in a single sample well. Adding a secondary assay for an additional level of multiplexing (i.e., a third data point) enables more information to be obtained per well. Having a built-in control for assay normalization helps to correct for well-to-well and day-to-day variability with a cell-based system. The assays presented here are compatible with standard instrumentation found in academic labs as well as in higher throughput screening environments.

References

1. Riss, T.L. and Moravec, R.A. (2004) *ASSAY Drug Dev. Technol.* **2**, 51–62.
2. Niles, A. (2007) *Anal. Biochem.* **366**, 197–206.
3. Fan, F. and Wood, K.V. (2006) *ASSAY Drug Dev. Technol.* **5**, 127–36.
4. Niles, A. *et al.* (2007) *Cell Notes* **18**, 15–20.
5. Zhang, J.H. *et al.* (1999) *J. Biomol. Screen.* **4**, 67–73.

Ordering Information

Product	Size	Cat.#
CytoTox-Glo™ Cytotoxicity Assay*[b,c]	10 ml	G9290
MultiTox-Glo Multiplex Cytotoxicity Assay*[b,c]	10 ml	G9270
MultiTox-Fluor Multiplex Cytotoxicity Assay*[a]	10 ml	G9200
CytoTox-Fluor™ Cytotoxicity Assay*[a]	10 ml	G9260
CellTiter-Fluor™ Cell Viability Assay*[a]	10 ml	G6080
Caspase-Glo® 3/7 Assay*[b,c,d]	2.5 ml	G8090
Apo-ONE® Homogeneous Caspase 3/7 Assay	1 ml	G7792

For Laboratory Use. Additional sizes available.

[a]Patent Pending.

[b]U.S. Pat. Nos. 6,602,677 and 7,241,584, Australian Pat. Nos. 754312, 785294 and 2003300008 and other patents and patents pending.

[c]The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Pat. Nos. 5,583,024, 5,674,713 and 5,700,673.

[d]U.S. Pat. No. 7,148,030 and other patents pending.

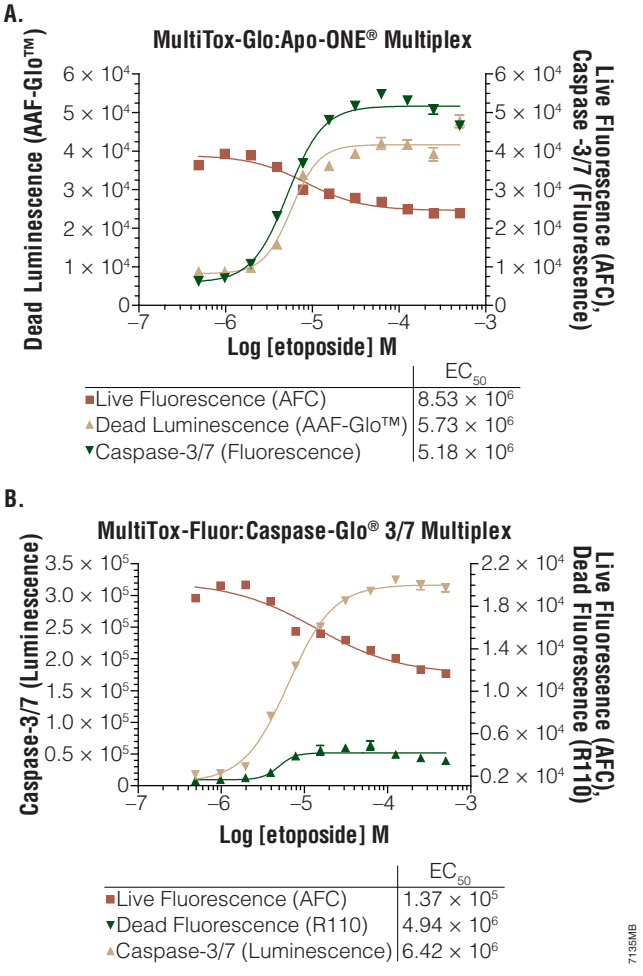

Figure 5. The MultiTox-Fluor and MultiTox-Glo Assays can be performed in multiplex with downstream assays, such as caspase activity assays.

Method. Using the protocols outlined in the flow charts (Figures 4 and 5), Jurkat cells were plated at 5,000 cells per well in a Corning Costar® (Cat.# 3705) 384-well plate. A serial 1:2 dilution of etoposide was made across the plate and cells were incubated with the treatment agent for six hours at 37°C, 5% CO_2.

MultiTox-Glo:Apo-ONE® Assay Multiplex Experiment. GF-AFC reagent was added, and plates were incubated for 30 minutes in 37 °C, 5% CO_2 before reading fluorescence on a Tecan Safire2™ plate reader. AAF-Glo™ reagent (dead-cell) was then added, and luminescence was read after a 20-minute incubation. Finally, to obtain caspase activity data, Apo-ONE® Reagent was added, cells were incubated 18 hours, and fluorescence was recorded.

MultiTox-Fluor:Caspase-Glo® 3/7 Assay Multiplex Experiment. MultiTox-Fluor reagent was added, and plates were incubated for 30 minutes before reading fluorescence on a Tecan Safire2™ plate reader. Next, Caspase-Glo® 3/7 Reagent was added; cells were incubated for 30 minutes, and luminescence was read.

Results. A dose-dependent decrease in live-cell signal is accompanied by a dose-dependent increase in dead-cell signal. Apoptosis data collected from the same sample show a dose-dependent increase in caspase-3/7 activity, suggesting that the cells are undergoing apoptosis.

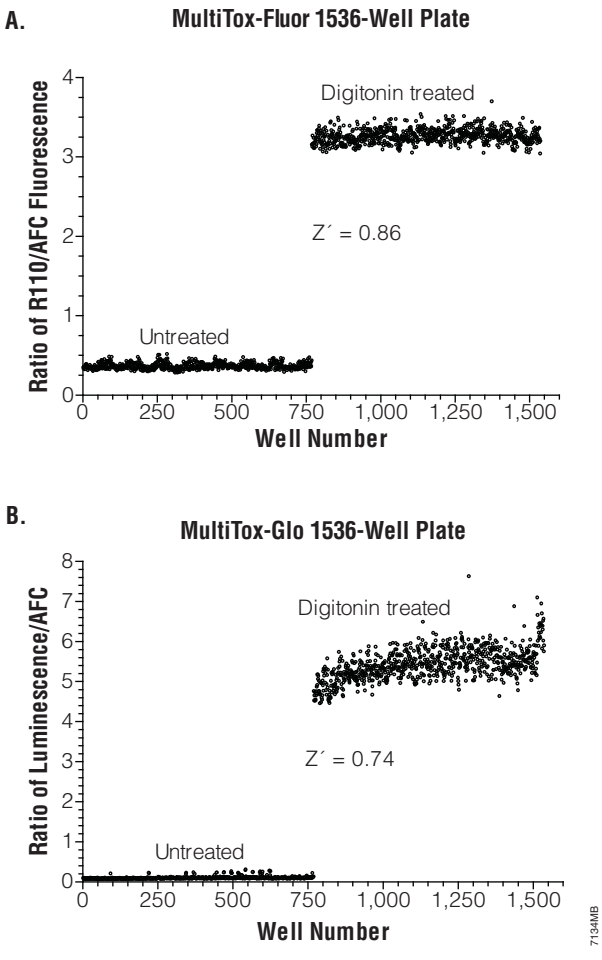

Figure 6. MultiTox-Fluor and MultiTox-Glo Assays are robust, high-quality assays yielding Z´-factor values above 0.7 in both 384-well (not shown) and 1536-well (shown) assay formats.

Method for the 384-Well Assay Format. Jurkat cells were maintained in suspension by tip mixing within the trough before transferring to the assay plate. Five thousand cells per well were dispensed into a Corning Costar® white plate (Corning Cat.# 3705) using the CyBi®-Well instrument. Cells in one half of the plate were treated with 100 µM ionomycin for two hours at 37 °C in 5% CO_2; untreated control cells in the other half of the plate received medium only.

Results. The 384-well format experiment produced a Z´-factor value of 0.79 for the MultiTox-Fluor Assay (data not shown) and 0.76 for the MultiTox-Glo Assay (data not shown).

Method for the1536-Well Assay Format. Jurkat cells were treated with 30 µg/ml digitonin and 2,500 cells per well were transferred to one half of a Corning white plate (Corning Cat.# 3937) using the CyBi®-Well instrument. Untreated cells were added to the other half of the plate. Fluorescent and luminescent signals were recorded with the Tecan Safire²™ microplate reader. Normalized results were obtained by determining the ratio of viable to nonviable cells for each experiment.

Results. The 1536-well format experiments produced a Z´-factor value of 0.86 for the MultiTox-Fluor Assay and 0.74 for the MultiTox-Glo Assay (data shown above).