Automation of Novel Assays for Cell-Based Multiplexing

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1. Abstract

We demonstrate the use of automation to perform high-throughput and ultra high-throughput cell-based multiplexing studies. We provide protocols and supporting data that demonstrate the automation synergies of the assays and CyBio CyBi®-Well hardware described here. The Promega MultiTox-Fluor™ and MultiTox-Glo™ are novel non-lytic reagents that have been developed to distinguish between viable and non-viable populations of cells in the same well. The ratio of live and dead cells can be used to normalize data in order to reduce error associated with cell dispensing or clumping. Using independent cell viability and toxicity readouts within the same well serves as an internal control and helps identify errors from interferences due to test compounds or medium components.

We also demonstrate how the MultiTox assays can be further multiplexed with a downstream assay, such as a Promega luminescent assay or other spectrally distinct fluorescent assay method, such as those measuring caspase activation, reporter activity, or other methods for measuring cell viability. Specifically, we show how these novel assays can be further multiplexed with downstream apoptosis assays in order to determine mode of action effects.

2. MultiTox-Fluor Assay Concept

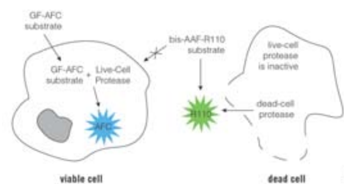


Figure 1. Biology of the MultiTox-Fluor Multiplex Cytotoxicity Assay. The MultiTox-Fluor Assay simultaneously measures two protease activities; one is a marker for cell viability, the other a marker for cytotoxicity. The live cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant, peptide substrate (glycyl-phenylalanyl-aminofluorocoumarin; GF-AFC, ex. 400nm, em. 505nm). The substrate enters intact cells where it is cleaved by the live-cell protease to generate a fluorescent signal proportional to the number of living cells. The live cell protease becomes inactive upon loss of membrane integrity and leakage into the surrounding culture medium. A second fluorogenic, cell-impermeant peptide substrate (bis-alanylalanyl-phenylalanyl-rhodamine 110; bis-AAF-R110, ex. 485nm, em. 520nm) is used to measure dead cell protease activity released from cells that have lost membrane integrity.

3. MultiTox-Glo Assay Concept

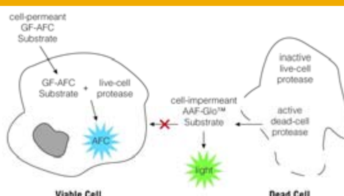
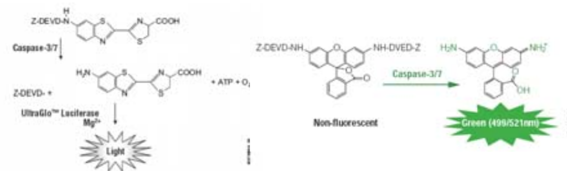


Figure 2. Biology of the MultiTox-Glo Multiplex Cytotoxicity Assay. The MultiTox-Glo Assay simultaneously measures two protease activities; one is a marker for cell viability, the other a marker for cytotoxicity. The live cell protease activity is restricted to intact viable cells and is measured using a fluorogenic, cell-permeant, peptide substrate (glycyl-phenylalanyl-aminofluorocoumarin; GF-AFC, ex. 400nm, em. 505nm). The substrate enters intact cells where it is cleaved by the live-cell protease to generate a fluorescent signal proportional to the number of living cells. The live cell protease becomes inactive upon loss of membrane integrity and leakage into the surrounding culture medium. A second luminescent, cell-impermeant peptide substrate (bis-alanylalanyl-phenylalanyl; AAF-Glo™, luminescent) is used to measure dead cell protease activity released from cells that have lost membrane integrity.

4. Caspase-Glo® 3/7 and Apo-ONE® Assay Concepts



A. Caspase-Glo 3/7 Assay

B. Apo-ONE Assay

Figure 3. Assay concept for Caspase-Glo 3/7 and Apo-ONE apoptosis assays. The Caspase-Glo 3/7 Assay (panel A) measures caspase -3 and -7 activity via cleavage of a luminogenic DEVD-luciferin substrate. Free luciferin reacts with firefly luciferase to generate light. Light output is proportional to caspase -3/7 activity. The Apo-ONE Assay (panel B) also measure caspase-3 and -7 via cleavage of a profluorescent substrate (bis-(N-CBZL- aspartyl-L-glutamyl-L-valyl-L-aspartic acid amide; Z-DEVD-R110). Upon cleavage of the substrate, the R110 leaving group (ex. 485nm, em. 520nm) becomes intensely fluorescent. Fluorescence is proportional to caspase activity.

5. Multiplexed Cell-Based Assay Protocols

Performing MultiTox Assays Stand Alone (384- and 1536-Well Protocols)

(1536-well volumes listed in parentheses)

MultiTox-Fluor Assay

Step	Description
1. Cells + treatment	20µl (4 µl) by CyBi-Well.
2. Incubation @ 37ºC	Pre-determined according to cell model used.
3. Reagent* addition	20µl (4 µl) of MultiTox-Fluor by CyBi-Well.
4. Incubation @ 37ºC	30 minutes minimum, up to maximum of 3 hours.
5. Read plate	Record live cell signal (ex. 400nm, em. 505nm) and dead cell signal (ex. 485nm, em. 520nm).

* MultiTox-Fluor reagent prepared by adding 10 µl AFC and R110 substrates to 10 ml of assay buffer.

MultiTox-Glo Assay

Step	Description
1. Cells + treatment	20µl (4µl) by CyBi-Well.
2. Incubation @ 37ºC	Pre-determined according to cell model used.
3. Reagent addition	10µl (2µl) of GF-AFC substrate by CyBi-Well.
4. Incubation @ 37ºC	30 minutes.
5. Read plate	Record live cell signal (ex. 400nm, em. 505nm).
6. Reagent addition	10µl (2µl) AAF-Glo substrate by CyBi-Well.
7. Incubation @ RT	20 minutes.
8. Read plate	Record dead cell signal (luminescence).

Performing MultiTox Assays With Downstream Assays (384-Well Protocol)

MultiTox-Fluor Assay

Step	Description
1. Cells + treatment	20µl by CyBi-Well.
2. Incubation @ 37ºC	Pre-determined according to cell model used.
3. Reagent** addition	5µl of MultiTox-Fluor by CyBi-Well.
4. Incubation @ 37ºC	30 minutes minimum, up to maximum of 3 hours.
5. Read plate	Record live cell signal (ex. 400nm, em. 505nm) and dead cell signal (ex. 485nm, em. 520nm).
6. Apoptosis Reagent	25µl of Caspase-Glo 3/7 by CyBi-Well.
7. Incubate @ RT	30 minutes - 3 hours after addition.
8. Read plate	Record luminescence.

** MultiTox-Fluor reagent prepared by adding 10µl AFC and R110 substrates to 2.5ml of assay buffer.

MultiTox-Glo Assay

Step	Description
1. Cells + treatment	20µl by CyBi-Well.
2. Incubation @ 37ºC	Pre-determined according to cell model used.
3. Reagent addition	10µl of GF-AFC substrate by CyBi-Well.
4. Incubation @ 37ºC	30 minutes.
5. Read plate	Record live cell signal (ex. 400nm, em. 505nm).
6. Reagent addition	10µl AAF-Glo substrate by CyBi-Well.
7. Incubation @ RT	20 minutes.
8. Read plate	Record dead cell signal (luminescence).
9. Apoptosis Reagent	40µl of Apo-One by CyBi-Well.
10. Incubate @ RT	30 minutes - 18 hours after addition.
11. Read plate	Record fluorescence (ex. 485nm, em. 520nm).

6. CyBio CyBi-Well 384/1536

Figure 4. CyBi-Well 384/1536 with Stackers. The CyBi-Well 384/1536 is a high-precision, simultaneous pipetting instrument that includes a stationary pipetting head with 384 channels and disposable tips, a plate carrier to move microplates to and from the pipettor, a plate lifter for moving plates up to the tips and a stage for indexing plates to access 1536-well plate quadrants. Disposable tip cartridges are loaded into the pipetting head and tips are sealed independently against a gasket for optimum pipetting performance. The CyBi-Well has a modular design which enables its use as a benchtop pipettor for assay development (for example in addition which stackers or a CyBi-Drop), or can be incorporated into larger screening systems for higher throughput.

7. Z´-Factor in 384- and 1536-Well Formats

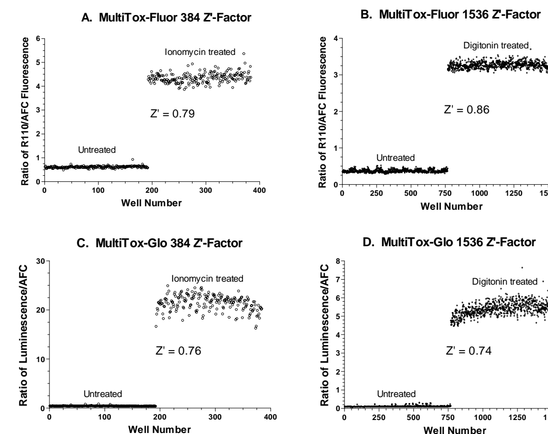


Figure 5. Using a ratiometric calculation for Z'-Factor improves results. Cell plating and reagent dispensing can introduce variability that can adversely affect data from a cell-based experiment. By taking a ratiometric approach to data analysis, one can normalize for this variability from well to well.

The Z´-factor (Zhang, *et al.*) data shown here are for normalized results obtained by determining the ratio of non-viable to viable cells for each experiment. For the MultiTox-Fluor Z´-factor experiments (Panels A and B), raw signal from the R110 substrate (dead cells; ex. 485nm, em. 520nm) is divided by raw signal obtained from the GF-AFC substrate (live cells; ex. 400nm, em. 505nm). For the MultiTox-Glo Z´-factor experiments (Panels C and D), raw signal from the AAF-Glo substrate (dead cells; luminescence) is divided by raw signal obtained from the GF-AFC substrate (live cells; ex. 400nm, em. 505nm). All fluorescent and luminescent readings were taken with the Tecan Safire2™ microplate reader. Z´-factor results for all normalized results are > 0.5, indicating excellent assay performance.

For the 384-well experiments, 5,000 Jurkat cells were dispensed per well with CyBi-Well, followed by treatment of one-half the plate with 100µM ionomycin or medium for two hours at 37ºC/5% CO_2. For the 1536-well experiments, 2,500 Jurkat cells were treated with 30µg/ml of digitonin and plated with CyBi-Well. To the other half of the plate, cells were plated with CyBi-Well and treated with medium.

Data shown here was plotted using Graphpad Prism® software.

8. Multiplexing MultiTox With Downstream Assays

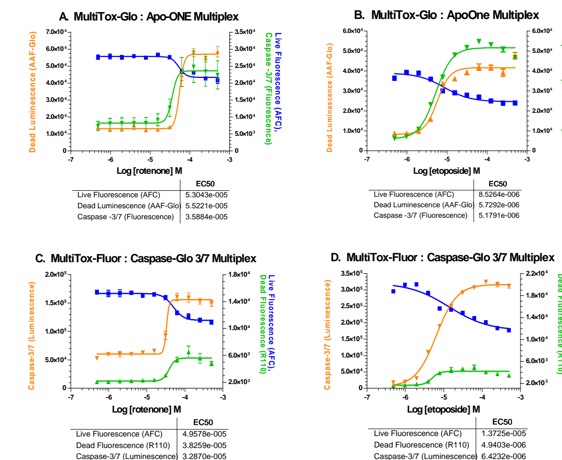


Figure 6. The MultiTox Assays can be multiplexed with apoptosis assays. The data presented here show the type of information that can be obtained by multiplexing MultiTox-Fluor and MultiTox-Glo assays with Caspase-Glo 3/7 and Apo-ONE assays, respectively. In all instances, the MultiTox data show that with increasing concentration of drug, there is a decrease in cell viability with a corresponding increase in cell death. The apoptosis assays confirm that there is an increase in caspase-3/7 activity, suggesting that the cells are undergoing apoptosis. Both reagent combinations show comparable results. Data were plotted using Graphpad Prism software.

For the MultiTox-Glo with Apo-ONE multiplexes shown in Panels A and B, 5,000 Jurkat cells were plated with CyBi-Well, followed by the addition of a serial 1:2 dilution of either rotenone or etoposide. The plate was incubated for 6 hours at 37ºC in 5% CO_2. The additions of GF-AFC and AAF-Glo reagents were performed following the multiplexing protocols listed previously, and fluorescent and luminescent signal was recorded with the Tecan Safire2. Apo-ONE Reagent was added to the plate and allowed to incubate at room temperature. Fluorescence was recorded with the Tecan Safire2 at 2 hours and 18 hours post reagent addition. The Apo-ONE data shown here is from the 18 hour read.

For the MultiTox-Fluor with Caspase-Glo multiplexes shown in Panels C and D, 5,000 Jurkat cells were plated with CyBi-Well, followed by the addition of a serial 1:2 dilution of either rotenone or etoposide. The plate was incubated for 6 hours at 37ºC in 5% CO_2. MultiTox-Fluor reagent was then added in 5µl volumes. The plate was mixed and incubated at 37ºC in 5% CO_2 for 30 minutes. Fluorescence was read on a Tecan Safire2. Caspase-Glo 3/7 reagent was then added, followed by a plate mix and room temperature incubation for 30 minutes. Luminescence was subsequently recorded with the Tecan Safire2.

9. Summary

Flexibility of MultiTox Assays
- Simultaneous measurement of live and dead cells in one well
- Obtain more data by multiplexing with downstream assays

Increased data confidence
- Built-in control for normalizing well-to-well and day-to-day variability

Automation-friendly
- Reagents compatible with liquid handling instrumentation
- Assays are scaleable to 384- and 1536-well formats

For more information on the MultiTox assays, please see:
"A Luminescent Cytotoxicity Assay for Drug Discovery" by A. Niles, *et al.*, SBS Poster #PST1J006 or Promega Booth #811.

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