MULTIPLEXED VIABILITY, CYTOTOXICITY AND APOPTOSIS ASSAYS FOR CELL-BASED SCREENING

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Previously, we introduced the MultiTox-Fluor Multiplex Cytotoxicity Assay technology (1) as a novel means for determining the relative number of live and dead cells in culture. In this article, we demonstrate that the assay technology is sufficiently scalable, sensitive and robust to be multiplexed with other downstream assays to increase the quality of screening data.

Introduction

Recent advances in combinatorial synthesis chemistries have led to both larger and more focused libraries of new chemical entities. The challenge continues to be testing these compounds for biological effects while maintaining cost economy. Although increasing throughput has helped efficiency, a number of factors shape the quality of the data derived from a screen. Ultimately, this quality dictates the probability of identifying active compounds worthy of further characterization.

Cell-based assay applications are being adopted with increasing frequency by drug discovery programs because cell systems are often inherently predictive of in vivo responses. For example, simple cell-based systems can be used to address potential compound toxicity, metabolic degradation or impaired permeability. Engineered or phenotype-specific lines also can be exploited to screen for compounds that modulate specific signaling cascades or regulatory elements. These data are not available from biochemical systems. However, cell-based systems also have clear limitations with respect to biological variability. This variability arises from various sources including unexpected toxicity or lack of uniformity in cell number. This variability can complicate data analysis and quality. Here we outline measures that improve screening data, leading to greater operator confidence.

Assay Principle and Chemistry

The MultiTox-Fluor Cytotoxicity Assay[(a)] simultaneously measures the relative number of live and dead cells in culture by detecting changes in cell membrane integrity. The assay reagent consists of two distinct fluorogenic peptide substrates that are introduced into the culture well via a physiologically balanced buffer (Figure 1). Viable cells are detected when the cell-permeant substrate (GF-AFC) enters the cell and is cleaved by a conserved and constitutive proteolytic activity resulting in liberated fluorophore. This live-cell protease activity is proportional to cell number, is restricted to viable cells and cannot be measured in dead-cell populations. The cytotoxic population

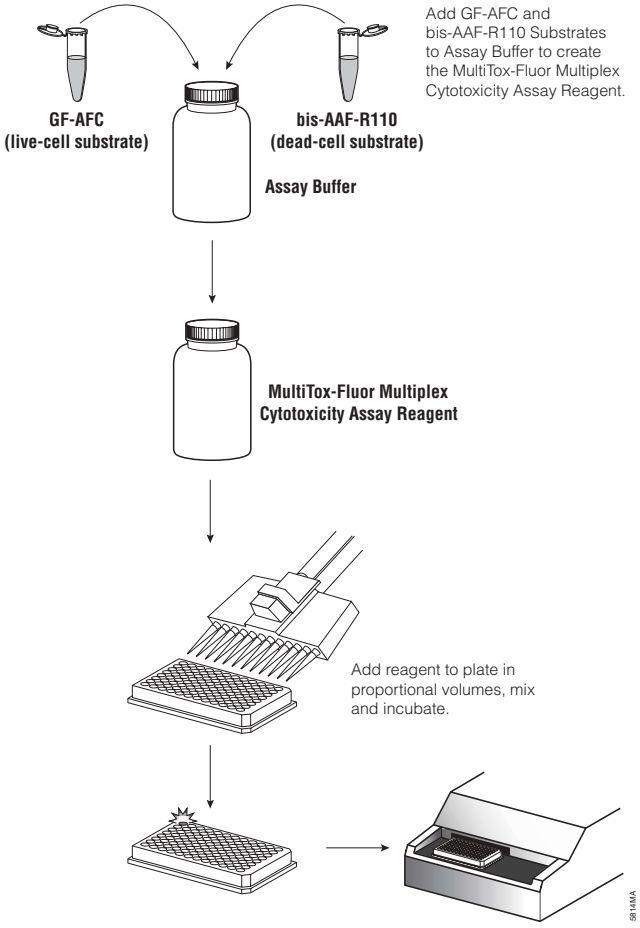

Figure 1. Schematic diagram of the MultiTox-Fluor Multiplex Cytotoxicity Assay. The MultiTox-Fluor Multiplex Cytotoxicity Reagent is created by adding the fluorogenic peptide substrates to the assay buffer. This reagent can then be added to a multiwell plate. After at least 30 minutes of incubation at 37˚C, the resulting fluorescent signal may be measured.

is measured by the presence of a dead-cell proteolytic activity that results from cells leaking their cytoplasmic contents into cell culture medium after membrane damage. The dead-cell substrate (bis-AAF-R110) is not cell permeant, so no appreciable signal is generated by intact viable cells.

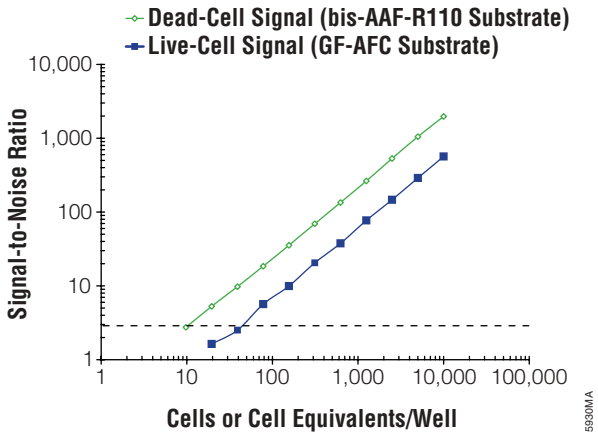

Figure 2. Statistical sensitivity of the MultiTox-Fluor Assay. Jurkat cells were twofold serially diluted from 10,000 to 10 cells/well in an opaque-walled, 96-well plate. RPMI 1640 with 10% fetal bovine serum served as the background control. Medium or medium containing detergent was added to matched wells to simulate cytotoxicity. MultiTox-Fluor Assay Reagent was added in an equal volume, and data were collected on a BMG POLARstar plate reader after 30 minutes of incubation at 37°C. Signal-to-noise determinations were calculated by dividing the net RFU (raw values minus background) by the standard deviation of the background at each fluorescence wavelength. The dotted line represents the statistical level of sensitivity (2). The best-fit lines for the dead- and live-cell measurements have r^2 values of 0.9998 and 0.9991, respectively.

The liberated fluorophores generated from these respective activities can then be measured using conventional multiwell fluorometers. This is possible because the optimal excitation and emission spectra for the liberated fluors are sufficiently separated to allow multiplexed live- and dead-cell measurements.

Sensitive, Scalable and Fast

High-density, cell-based formats require high assay sensitivity. In most cases, the MultiTox-Fluor Assay offers statistical sensitivities approaching the level of the CellTiter-Glo® Assay (which measures ATP-levels) after only 30 minutes of incubation at 37ºC (Figure 2). Unlike ATP-based assays, which require cell lysis to liberate ATP, the MultiTox-Fluor Cytotoxicity Assay sensitivity is achieved without affecting viability. This high sensitivity allows the reagent to be employed in 384- and 1536-well plate formats where both cell number and well volumes are significantly reduced compared to 96-well formats (Figures 3 and 4).

The final reagent concentration required for the MultiTox-Fluor Cytotoxicity Assay is also flexible to accommodate well volume restrictions when conducting other sequentially multiplexed assays within the same well. For instance, a concentrated reagent can be prepared and delivered in a 1/10th volume when multiplexing with other assays.

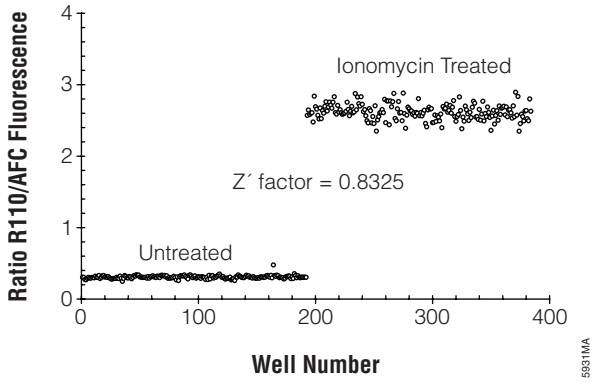

Figure 3. Demonstration of performance in a 384-well primary necrosis model. Jurkat cells were plated at a density of 5,000 cells per well in 10µl volumes using a CyBio CyBi® Well 384/1536 automated dispenser. Ionomycin was diluted in RPMI 1640 with 10% fetal bovine serum and added in an additional 10µl volume to half of the plate to a final concentration of 50µM. Complete medium was added to the other half of the plate, and the plate was incubated for 5 hours at 37°C. MultiTox-Fluor Assay Reagent was added in 20µl volumes and incubated for 30 minutes prior to fluorescence measurement using a Tecan Safire²™ Multichannel Monochromator reader. Z´-factor values were determined by using the ratio of fluorometric signals for each well rather than comparing average signals for control populations (2).

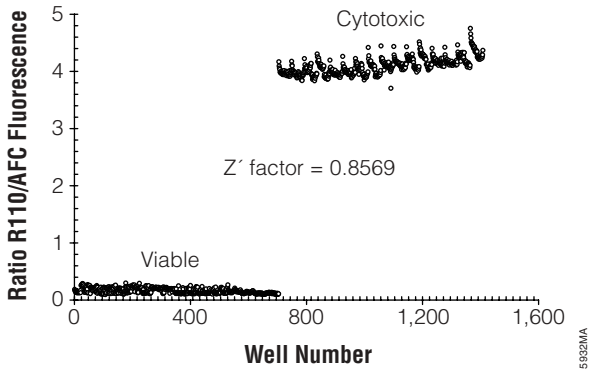

Figure 4. Demonstration of performance in a 1536-well format. Jurkat cells were adjusted to 625,000 cells/ml in RPMI 1640 with 10% fetal bovine serum. The pool was divided, and one fraction gently sonicated to simulate 100% cytotoxicity. Cells or lysate were delivered in 4µl volumes (2,500 cell equivalents) using a Deerac Fluidics Equator™ automated dispenser. MultiTox-Fluor Assay Reagent was added in an additional 4µl volume and the plate incubated at 37°C for 2 hours prior to measuring fluorescence using a Tecan Safire²™ Multichanel Mononchromator reader. Z´-factor values were calculated by using the ratio of fluorometric signals for each well rather than comparing average signals for control populations (2).

The Ratiometric Response

The MultiTox-Fluor Cytotoxicity Assay measures two independent but inversely correlated protease activities as markers of cellular viability and cytotoxicity. When used in combination, these measures can complement each other and provide a ratiometric value that is useful for counter-

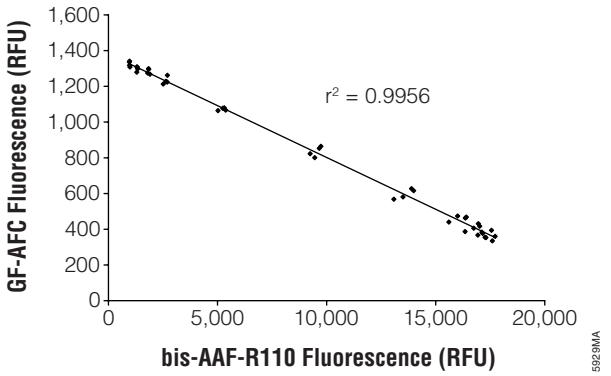

Figure 5. The inverse proportionality of the ratiometric response. Jurkat cells were adjusted to 100,000 cells/ml in RPMI 1640 with 10% fetal bovine serum, then divided into two pools. One fraction was treated by sonication to simulate cytotoxicity, the other left untreated. The fractions were blended in various ratios to create viabilities from 100% to 0%. 100µl of each blend was added to wells of a 96-well plate. Medium-only served as background control. After 30 minutes of incubation at 37°C, fluorescence was measured using a BMG POLARstar plate reader. Net fluorescence values (minus cell-free background) from both wavelengths were plotted against each other for each well.

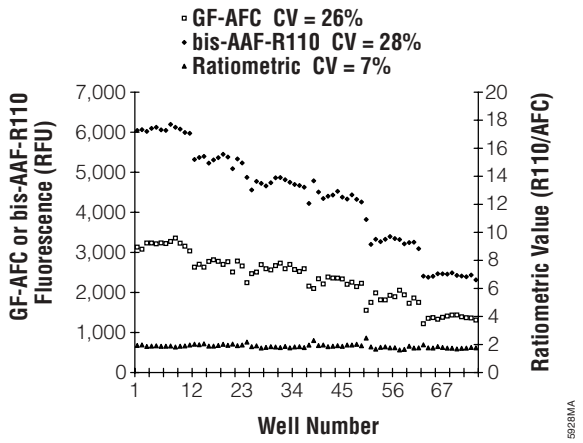

Figure 6. Improving assay precision by ratiometric means. A pool of Jurkat cells was divided, and one fraction was subjected to sonication to simulate cytotoxicity. The two fractions were combined to make a 50% viable pool. To simulate the effects of cellular clumping, the pool was diluted with additional RPMI 1640 with fetal bovine serum and delivered to wells of a 96-well plate at calculated densities of 12,500 (wells 1–12), 11,000 (wells 13–24), 10,000 (wells 25–36), 9,000 (wells 37–48), 7,500 (wells 49–60) and 5,000 cells/well (wells 61–72) in 100µl volumes. MultiTox-Fluor Assay Reagent was added in an equal volume to each well, and data were collected after 30 minutes of incubation at 37°C. The raw data collected at the AFC and R110 wavelengths were plotted against well number. The quotient of values derived from the same data is also represented. The coefficient of variation percentage is derived from the standard deviation of each data set divided by the average signal x 100.

confirmation of the result (Figure 5). In other words, if the viability measurement is low compared to an untreated control, the cytotoxicity measurement should be high compared to the untreated control (and vice versa). Divergence from the ratiometric relationship with any single compound or treatment may occur in three experimental situations: a proliferative event in the absence of cytotoxicity, compound interference with one of the fluorometric measures, or dead-cell enzyme activity decay over long exposure periods. Proliferation will demonstrate an increase in only the live-cell signal with respect to the control. Fluorescence interference will demonstrate either a disproportional increase or decrease in the signal. However, because of the spectral distance between the respective fluorophores' excitation and emission spectra, the likelihood of both markers being affected by an autofluorescent compound would be statistically rare (3). Finally, the dead-cell response may be underestimated in cases of primary necrosis or rapid apoptosis induction, because the marker demonstrates an enzymatic half-life of about nine hours after cell death. Nevertheless, the duality of the measures allows "flagging" of problematic data points, whereas single-parameter cytotoxicity assay measures may lead to potentially false-positive or -negative conclusions.

The ratiometric response is also useful for improving the precision of data resulting from variability in cell number due to cellular clumping or pipetting errors as well as differential growth patterns or edge effects in assay plates. This variability is particularly troublesome for single-point cytotoxicity assays and single-parameter assays that measure responses such as caspase induction potential or genetic reporter activity. The risk is that the data set from these screens may indicate statistically significant increases or decreases in activity when they are in fact false-negative or -positive. Because of the ratiometric proportionality of MultiTox-Fluor Cytotoxicity Assay, response variability arising from cell number differences in cytotoxicity assays can be resolved simply by using the quotient of dead-cell and live-cell values for each well (Figure 6). In some instances, data from primary activity assays can be normalized by first measuring the relative number of remaining live or dead cells in culture prior to adding the second reagent (Figure 7). Care should be exercised with such normalization, as differences in pharmacokinetic induction rates (and activity) may precede changes in cellular viability.

Increasing Content

The true cost of a screening effort is typically more than time, assay reagents and other consumable expenses. Compound usage and the informational value of compound characterization contribute to screening costs. Multiplex assays extract the valuable data and reduce library consumption by eliminating parallel assays. By multiplexing the MultiTox-Fluor Cytotoxicity Assay with specific cell-response assays, you not only reduce false-negative and -positive determinations, but also gain information about

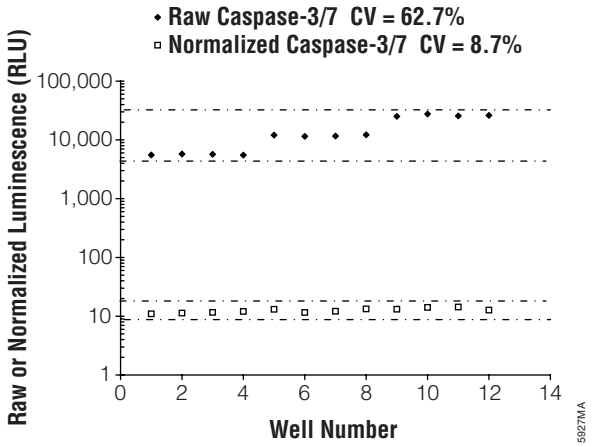

Figure 7. Primary caspase activity response data can be normalized by viability values. U266 cells were seeded at 5,000 (wells 1–4), 10,000 (wells 5–8), and 20,000 cells/well (wells 9–12) in 50µl of RPMI 1640 with 10% fetal bovine serum. Staurosporine was diluted in medium and added to 2µM final concentration with an additional 50µl volume. The plate was incubated for a period of 6 hours at 37°C to allow caspase activation by apoptosis induction. MultiTox-Fluor Assay Reagent was made by adding 10µl of each substrate to 1.0ml of assay buffer. The reagent was then added in 10µl volumes to the wells, and data were collected after 30 minutes of incubation at 37°C. Caspase-Glo® 3/7 Reagent was added in 100µl volumes, and luminescence was measured after 30 minutes. Raw Caspase-Glo® 3/7 response data from the varying cell number wells are plotted together with the same data normalized by live cell response values. The coefficient of variation for each was calculated by dividing the standard deviation of the data set by the average signal x 100 to arrive at a percentage.

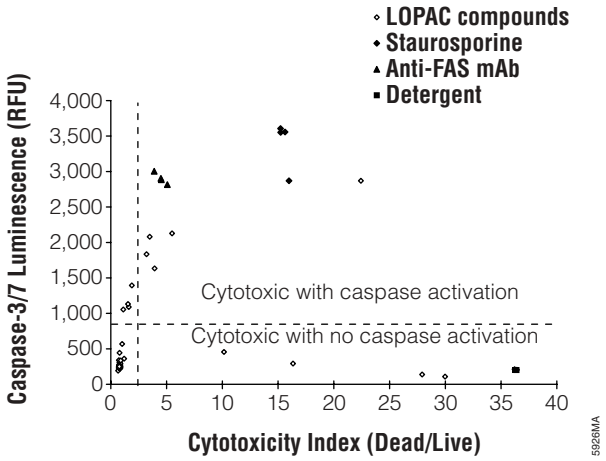

Figure 8. Creating a cytotoxicity index using MultiTox-Fluor Assay and a luminescent caspase-3/7 assay. Jurkat cells at a density of 5,000 cells/well in 50µl volumes were exposed to 80 compounds from the LOPAC library (plate 4) in additional 50µl volumes for a final concentration of 10µM. Known apoptotic inducers, anti-FAS mAb at 100ng/ml (black triangles) and staurosporine at 2µM (black diamonds) served as caspase-activation controls. Detergent (black square) served as a primary necrosis control. The plate was incubated at 37°C for an 8-hour exposure period. MultiTox-Fluor Reagent was prepared and added as described in Figure 7, and fluorescence was measured after 30 minutes at 37°C. Caspase-Glo® 3/7 Reagent was added in 100µl volumes and luminescence measured after 30 minutes. A "cytotoxicity index" was established for each compound by dividing the dead-cell value by the live-cell value. The resulting data were plotted v. raw luminescence and partitioned into quadrants based on thresholds established from untreated cell values. Compounds that kill cells but do not induce caspase activation are clearly distinguished from compounds that induce caspase activation and are cytotoxic. Compounds that induce caspase activation but show no apparent cytotoxicity reflect early stage apoptosis.

inherently flawed or problematic compounds. For instance, compounds that induce a cytotoxic response in the absence of caspase activation are therapeutically unattractive (Figure 8). These "frequent hitters" then can be culled from the collection to reduce the throughput burden and strengthen the quality of future data.

Summary

The MultiTox-Fluor Multiplex Cytotoxicity Assay has many attributes that make it useful for cell-based assay screening. Use of the assay not only indicates compound effects on cellular viability but can also improve data quality by improving assay precision, detecting false-positives and -negatives, and by increasing content on a per well basis by multiplexes with specific response assays. This homogeneous, "add-mix-measure" assay is readily scalable for HTS applications but can be used at any stage throughout potency and lead selection testing. ■

References

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3. Grant, S. *et al.* (2002) *J. Biomol. Screen.* **7**, 531–40.

Protocol

MultiTox-Fluor Multiplex Cytotoxicity Assay Technical Bulletin #TB348
(www.promega.com/tbs/tb348/tb348.html)

Ordering Information

Product	Size	Cat.#
MultiTox-Fluor Multiplex Cytotoxicity Assay	10ml	G9200
	5 × 10ml	G9201
	2 × 50ml	G9202

For Laboratory Use.

[a]Patent Pending.