MULTITOX-FLUOR MULTIPLEX CYTOTOXICITY ASSAY TECHNOLOGY

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Here we introduce the MultiTox-Fluor Multiplex Cytotoxicity Assay technology that allows the measurement of the relative number of live and dead cells in culture wells. This assay provides inversely proportional values of cytotoxicity and viability that are useful for normalizing data to cell number. The MultiTox-Fluor Reagent can also be multiplexed with other cell-based assays.

Introduction

Cell-based assays are important tools for contemporary biology and drug discovery because of their predictive potential for in vivo applications. However, the same cellular complexity that allows the study of regulatory elements, signaling cascades, or test compound bio-kinetic profiles can also complicate data interpretation by inherent biological variation. Therefore, researchers often need to normalize assay responses to cell viability after experimental manipulation.

A multitude of assay chemistries are currently employed to determine viability or cytotoxicity in cell-based assays (1). Viability measurements typically rely on either metabolic capacity or other biochemical markers not present after cell death. For instance, the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay and the CellTiter-Blue® Cell Viability Assay use live-cell tetrazolium or resazurin reduction, respectively, while CellTiter-Glo® Luminescent Cell Viability Assay measures ATP after reagent-mediated lysis to assess cell viability.

The MultiTox-Fluor Assay technology gives ratiometric, inversely proportional values of viability and cytotoxicity that are useful for normalizing data to cell number.

Many cytotoxicity assays directly assess the structural integrity of cell membranes by measuring the capacity to exclude dyes or retain cytoplasmic contents (2). Trypan blue and propidium iodide are classic examples of dyes that freely enter compromised or dead cells but do not stain the viable population. Enzyme markers like lactate dehydrogenase (LDH), which leak from the cytoplasm into culture medium upon cell death, can be conveniently measured using coupled enzymatic assays like the CytoTox-ONE™ Homogeneous Membrane Integrity Assay (3).

Although the existing assays for determining cellular viability or cytotoxicity are useful and cost-efficient methods, they have limits in the types of multiplexed assays that can be performed along with them. For instance, the CellTiter-Glo®

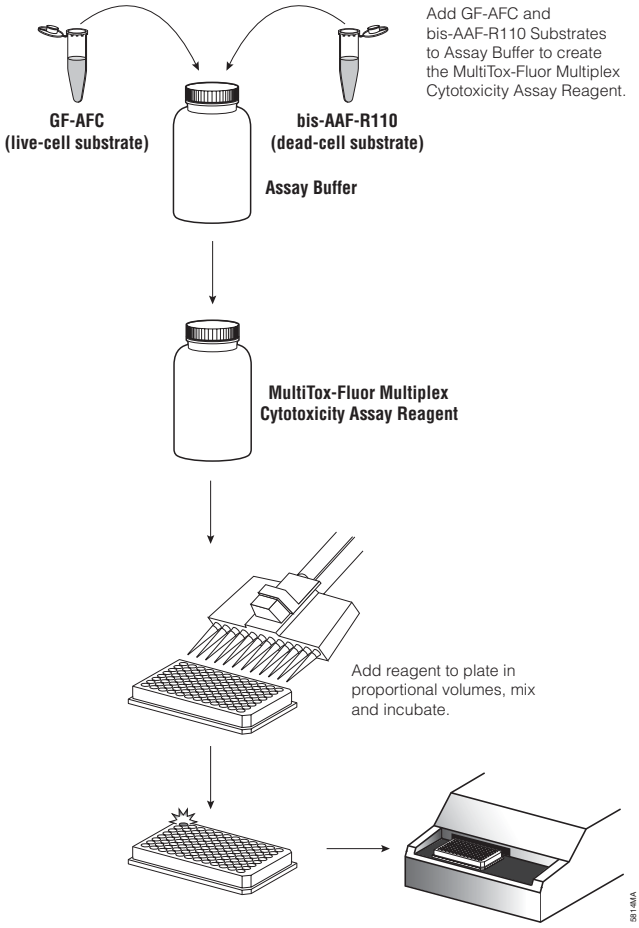

Figure 1. Schematic diagram of the MultiTox-Fluor Multiplex Cytotoxicity Assay. The MultiTox-Fluor Multiplex Cytotoxicity Reagent is created by adding the fluorogenic peptide substrates (GF-AFC, live-cell protease substrate and bis-AAF-R110, dead-cell protease substrate) to the assay buffer. This reagent can then be added to a multiwell plate. After at least 30 minutes of incubation at 37°C, the resulting fluorescent signals may be measured at an excitation of 400nm and an emission of 505nm, then at an excitation of 485nm and an emission of 520nm.

Assay is a lytic endpoint assay that is incompatible with other assays that measure beetle luciferase (e.g., reporter gene assays) or luciferin (e.g., luminescent caspase assays). Additionally, the resazurin chemistries used for viability and cytotoxicity measures cannot be combined in the same well

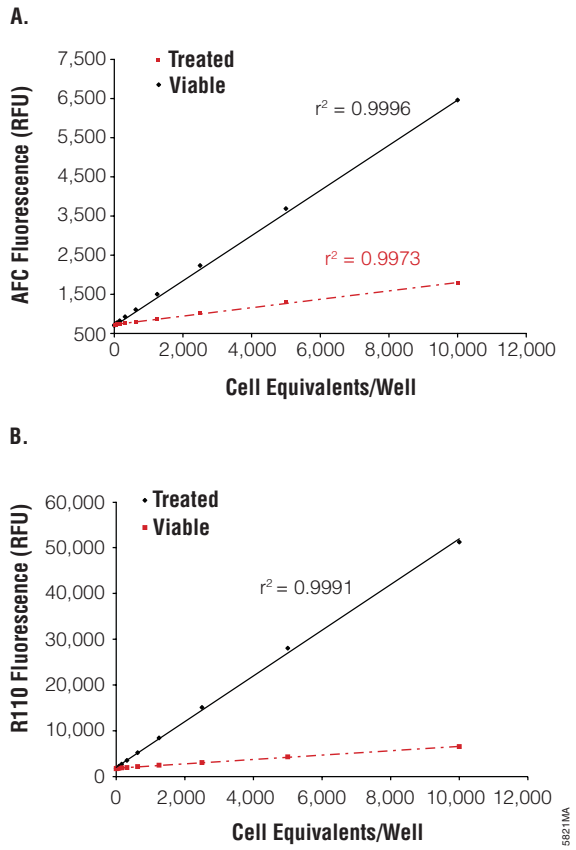

Figure 2. The MultiTox-Fluor Multiplex Cytotoxicity Assay technology measures two distinct proteolytic activities that are mutually restricted to either viable or compromised cells. A pool of Jurkat cells was divided into two fractions. One fraction was treated to simulate cytotoxicity, whereas the other was left untreated. Both fractions were twofold serially diluted in RPMI 1640 + 10% fetal bovine serum. Medium with serum served as the no-cell control. Single substrate reagents were created by adding either GF-AFC or bis-AAF-R110 Substrate to buffer. Each was added to cells and incubated for 30 minutes at 37°C prior to measuring fluorescence using a BMG PolarStar plate reader. **Panel A** represents the differential fluorescence profile of the live-cell substrate (GF-AFC Substrate). **Panel B** represents the differential fluorescence profile of the dead-cell substrate (bis-AAF-R110 Substrate).

because of spectral overlap and reagent formulation incompatibility. Furthermore, the absorbance profile of resazurin can significantly complicate additional multiplexed downstream applications by color quenching of fluorescence or luminescence.

Here we report the development of a homogeneous, single-addition reagent that allows the measurement of the relative number of live and dead cells in culture wells. This assay technology gives ratiometric, inversely proportional values of viability and cytotoxicity that are useful for normalizing data to cell number. Lastly, this reagent is compatible with additional fluorescent and luminescent chemistries.

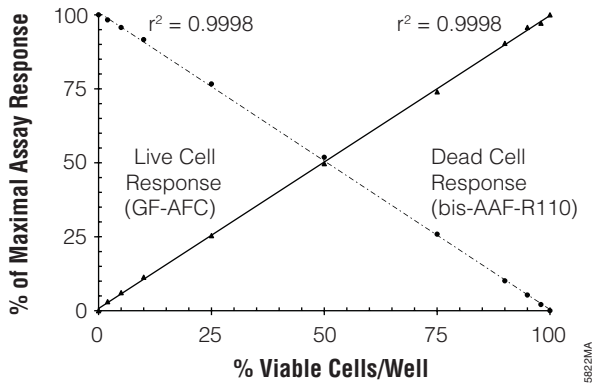

Figure 3. The ratiometric response obtained from the MultiTox-Fluor Assay technology. A pool of Jurkat cells was adjusted to 100,000 cells/ml, then divided into two fractions. One fraction was compromised by treatment to induce cytotoxicity; the other untreated. The two fractions were then combined in various proportions to simulate varying viabilities from 100 to 0%. 10,000 cell equivalents were added to each well in 100µl volumes, followed by an equal addition of the MultiTox-Fluor Assay Reagent. After brief orbital shaking, the plate was incubated at 37°C for 30 minutes before measuring fluorescence using the BMG PolarStar plate reader. The data were normalized as a percentage of the maximal response. The dashed line represents fluorescence generated in the presence of dead cells, whereas the unbroken line represents fluorescence in the presence of live cells.

Assay Design and Chemistry

The MultiTox-Fluor Multiplex Cytotoxicity Assay technology[a,b] is analogous to other assays that use membrane integrity changes to measure cell viability or cytotoxicity. However, instead of dye exclusion or LDH release, the MultiTox-Fluor Assay technology simultaneously measures two distinct protease activities as markers for cell viability or cytotoxicity. The live-cell protease activity is restricted to intact viable cells and is measured using the fluorogenic, cell-permeant, peptide substrate Gly-Phe-7-amino-4 trifluoromethyl coumarin (GF-AFC). The substrate enters intact cells where it is cleaved to generate a fluorescent signal proportional to the number of living cells. This live-cell protease activity marker becomes inactive upon loss of membrane integrity and leakage into the surrounding culture medium. The second cell-impermeant, fluorogenic peptide substrate, bis-(Ala-Ala-Phe)-rhodamine 110 (bis-AAF-R110), is used to measure dead-cell protease activity that has been released from cells that have lost membrane integrity. Because bis-AAF-R110 Substrate is not cell-permeant, essentially no signal from this substrate is generated by intact viable cells.

These fluorogenic substrates are formulated in a physiological buffer and delivered to cell culture wells to measure both the viable and dead cell populations simultaneously in the same sample (Figure 1). After proteolytic hydrolysis of the peptide bond in GF-AFC Substrate by the “live-cell protease”, the

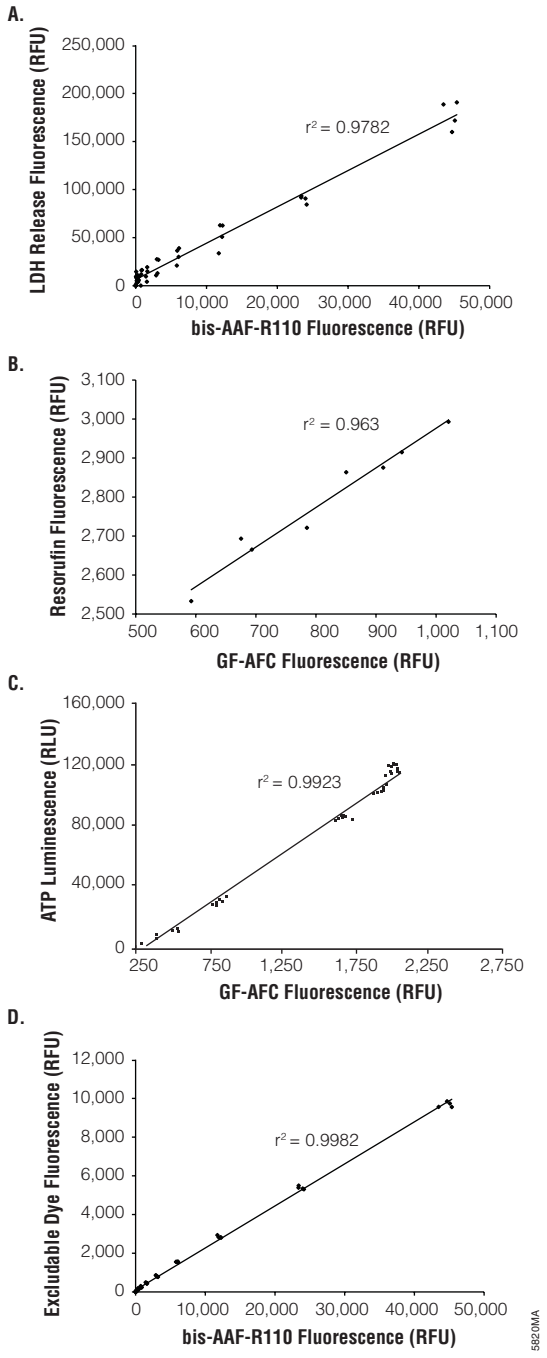

Figure 4. The MultiTox-Fluor Assay technology data correlate with other conventional measures of viability and cytotoxicity. U937, Jurkat, and HL-60 were subjected to various treatments, which induced different degrees of cytotoxicity. Parallel plates were prepared and cytotoxicity and viability determined by MultiTox-Fluor Assay technology, CytoTox*ONE*™ Assay (**Panel A**), CellTiter-Blue® Assay (**Panel B**), CellTiter-Glo® Assay (**Panel C**) or by ethidium homodimer incorporation (**Panel D**, Molecular Probes). Resulting fluorescence signals were plotted against MultiTox-Fluor Assay technology readings.

liberated AFC fluorophore can be measured using a fluorometer at an excitation of 400nm and emission of 505nm (4). The dead-cell protease cleaves bis-AAF-R110 to release the R110 fluorophore, which can be measured at an excitation of 485nm and emission of 520nm (5; Figure 2).

The greatest advantage derived from measuring the presence of both live and dead cells by the MultiTox-Fluor Multiplex Cytotoxicity technology is that the measurements are inversely proportional and complementary (Figure 3). Simply stated, when one fluorescence marker response is high, the other will be low when compared to viability or cytotoxicity controls. This provides a ratiometric response that can be used to normalize the data, irrespective of cell number. Ultimately, these independent measures serve as assay controls and can help identify and correct errors caused by pipetting, differential growth patterns or interference with assay chemistry.

Sensitivity

Traditional dye exclusion chemistries exhibit limited sensitivity. Although dye reduction assays represent an improvement in sensitivity because of continuous enzymatic cycling, the reactions may require multiple steps and involve product intermediates. The MultiTox-Fluor Multiplex Cytotoxicity Assay technology directly measures two different protease activities with rapid catalytic cleavage rates. This allows rapid accumulation of fluorescent product in cell culture. After a 30-minute incubation, even modest declines in viability (5%) can be measured in most cell types when using 10,000 cells/well (Figure 3). Increasing incubation times up to two hours often increases the signal window and detects cytotoxicity of as little as 2% of the population.

Correlation with Conventional Viability and Cytotoxicity Assays

The MultiTox-Fluor Multiplex Cytotoxicity Assay technology represents an entirely novel and previously undescribed method for measuring viability and cytotoxicity of cells in culture. However, because the assay is designed to measure distinct changes in membrane integrity, data derived from the assay correlate well with existing methodologies. Therefore, these measures of proteolytic activity mirror the data obtained from enzyme release, resazurin reduction, ATP quantitation or dye exclusion assays (Figure 4).

Additional Downstream Multiplex Possibilities

The MultiTox-Fluor Multiplex Cytotoxicity Assay Reagent can be delivered to wells in various volumes by adjusting the substrate concentration in the reagent. For instance, a 2X reagent can be prepared and added at a volume equal to the culture medium, or a 10X reagent can be prepared and added at a volume 1/10th that of the culture medium. This smaller volume addition provides flexibility and

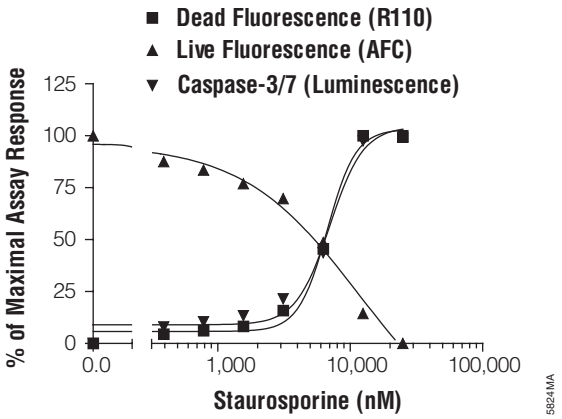

Figure 5. The MultiTox-Fluor Assay technology can be multiplexed with other assays. LN-18 cells were plated at a density of 10,000 cells per well in 50µl volumes of MEM + 10% fetal bovine serum and allowed to attach overnight. Staurosporine was twofold serially diluted and added to wells in 50µl volumes. The plate was incubated at 37°C in 5% CO_2 for 6 hours. MultiTox-Fluor Reagent was prepared as a 10X reagent and delivered to all wells in a 10µl volume. The plate was mixed by orbital shaking, then returned to the incubator for 30 minutes before reading the fluorescent signals on a BMG PolarStar plate reader. Caspase-Glo® 3/7 Reagent was then added in an additional 100µl volume, and resulting luminescence measured after a 10-minute incubation using the BMG PolarStar plate reader. The resulting signals were normalized to a percentage of the maximal response and plotted using GraphPad Prism® software.

Table 1. The MultiTox-Fluor Multiplex Assay technology was validated using cell lines representative of the diversity in the National Cancer Institute-60 (NCI-60) Panel.

Cell Line	Sex	Age	Histology	Source/Origin
HCT-116	M	>18	carcinoma	colon
HL-60 (TB)	F	36	promyelocytic	PBL leukemia
SK-MEL-28	M	51	melanoma	melanoma/skin
MCF-7	F	69	adenocarcinoma	mammary
PA-1	F	12	teratocarcinoma	ovary
ACHN	M	22	carcinoma	kidney
PC-3	M	62	adenocarcinoma	prostate
DU-145	M	69	carcinoma	prostate
NCI-H226	M	NA	squamous	lung
LN-18	M	65	glioblastoma	brain
HeLa	F	31	carcincoma	cervix
Jurkat	M	NA	T-cell leukemia	lymphocyte
Hek293	NA	fetal	transformed	kidney
HepG2	M	15	hepatocarcinoma	liver
NK-92CI	M	50	lymphoma	NK cell
U937	M	37	histocystic lymphoma	monocyte

accommodates the addition of other reagents that may be required for a second multiplexed assay for reporter activity, caspase activation, etc. Because the substrates are essentially colorless and inert with respect to influencing viability, a large number of multiplex options exist using spectrally distinct fluorophores or luminescent assays (Figure 5).

Cell-Specific Assay Responses

There is significant diversity with regard to morphology and lineage among cell lines used in routine biological research. Different cell lines have different reductive capacities or contain different levels of enzyme markers. These factors may influence assay incubation times or experimental sensitivity with conventional viability or cytotoxicity chemistries. The MultiTox-Fluor Multiplex Cytotoxicity Assay was validated using selected cell lines representative of the National Cancer Institute-60 Panel (6) and found to work well in various necrosis or apoptosis models (Table 1).

Summary

The MultiTox-Fluor Multiplex Cytotoxicity Assay uses simple, sensitive and scalable chemistries to determine the degree of viability and cytotoxicity within cell culture wells after experimental treatment. The live-cell and dead-cell proteolytic measures in the MultiTox-Fluor Multiplex Cytotoxicity Assay correlate well with conventional measures of cell viability and act as internal controls while providing a ratiometric response, which is useful for normalization purposes. Furthermore, the assay system provides the additional functionality and flexibility to enable multiplexing with other assays. ■

References

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Note: The MultiTox-Fluor Multiplex Cytotoxicity Assay is currently under development. Contact Promega Technical Services for more information.

Related Products

Product	Size	Cat.#
Apo-ONE® Homogeneous Caspase-3/7 Assay	1ml	G7792
	10ml	G7790
	100ml	G7791
CytoTox-ONE™ Homogeneous Membrane Integrity Assay	200–800 assays	G7890
	1,000–4,000 assays	G7891
CellTiter-Blue® Cell Viability Assay	20ml	G8080
	100ml	G8081
	10 × 100ml	G8082
CellTiter-Glo® Luminescent Cell Viability Assay	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573
Caspase-Glo® 3/7 Assay*	2.5ml	G8090
	10ml	G8091
	10 × 10ml	G8093
	100ml	G8092

*For Laboratory Use.

(a)This product is currently under development.

(b)Patent Pending.