USING PROTEASE BIOMARKERS TO MEASURE VIABILITY AND CYTOTOXICITY

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Introduction

Although several biomarkers have been described and employed for measuring viability and cytotoxicity in cell culture, none is without technical fault. We recently identified two new biomarker profiles for viability and cytotoxicity that circumvent many historical assay chemistry limitations and greatly facilitate multiplex measurements (1). These markers[(a)] have proteolytic activities associated with cell death or viability and can be measured in multiplex using either a single luminogenic substrate with sequential reads, a luminogenic substrate in combination with a fluorogenic substrate, or with two different fluorogenic substrates (2–4). Regardless of format, the assays for these markers generate large dynamic ranges with excellent linearity, providing unprecedented sensitivity in high-density formats (Figure 1).

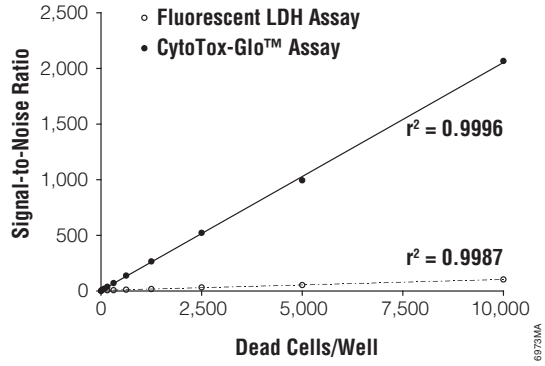
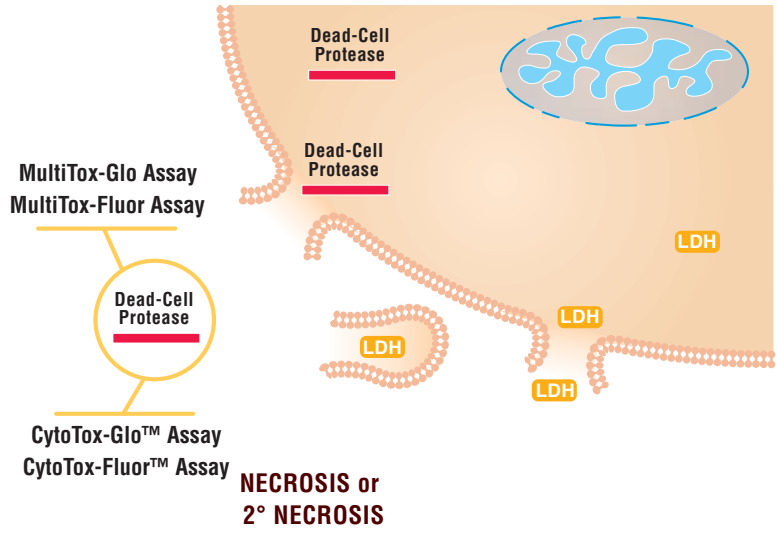
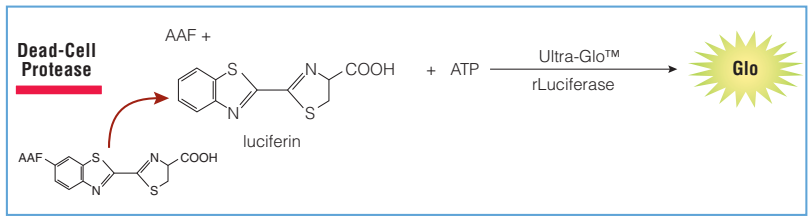


Figure 1. The CytoTox-Glo™ Assay detects small changes in viability because of its high sensitivity. This graph shows the superior signal-to-noise ratios of the CytoTox-Glo™ Assay compared to a fluorescent LDH assay.

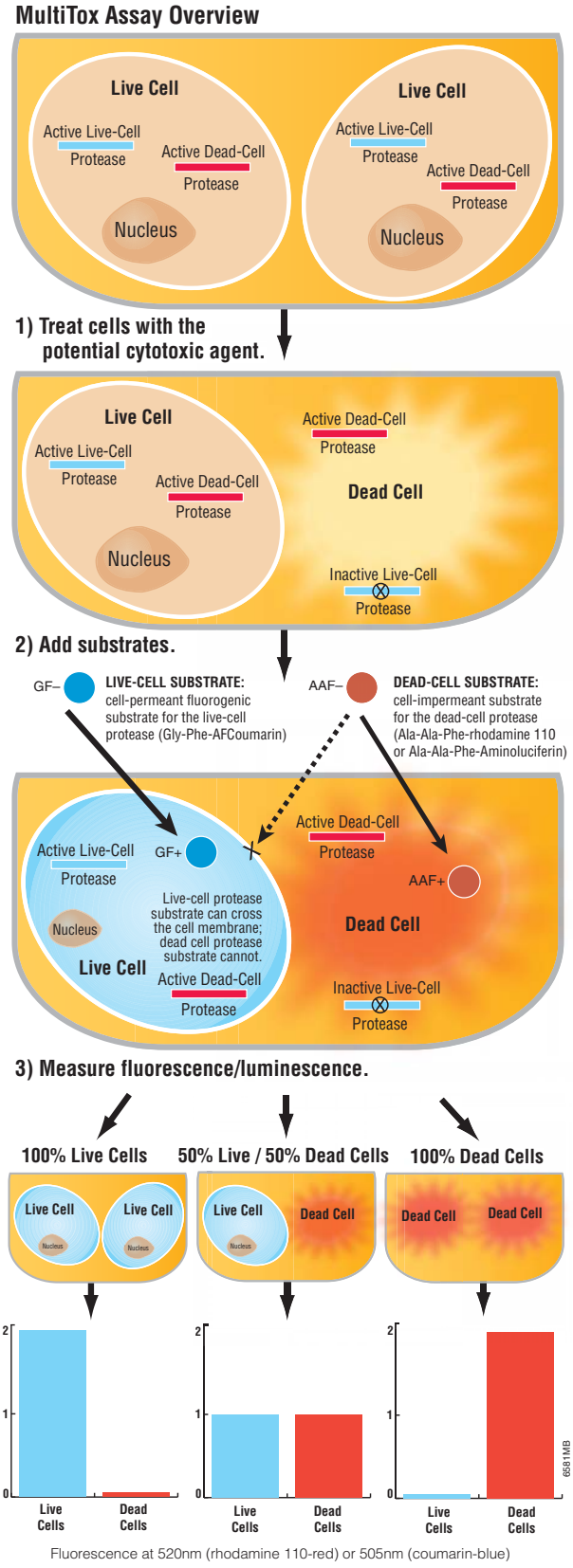


The CytoTox-Glo™ Cytotoxicity Assay is the most sensitive method for measuring cytotoxicity. The assay quantifies the extracellular activity of an intracellular protease (dead-cell protease) when the protease is released from membrane-compromised cells.



CytoTox-Glo™ Assay Chemistry.

Multiplex Cytotoxicity Assays



Fluorescence at 520nm (rhodamine 110-red) or 505nm (coumarin-blue)

CHALLENGE: Find enzymatic biomarkers for viability and cytotoxicity that are not modulated by any stimulus other than cytotoxicity and can discriminate betwen viable and nonviable populations in a proportional manner.

EXPERIMENT: We focused our peptide-based, protease activity screen on proteases with constitutive, homeostatic function. Substrates for known inducible proteases (e.g., caspases, granzymes, calpain, tryptase, etc.) were avoided until counterscreening. Each test fluorogenic substrate was exposed to a limiting dilution series of viable and nonviable cells to determine if it could select between viable and dead cells.

RESULTS: Two proteolytic profiles emerged from cell-based screening (Table 1):

- An activity restricted to viable cells using Gly-Phe-AFC. This activity (likely from distinct aminopeptidases) was significantly reduced in equivalent numbers of nonviable cells due to enzymatic lability.
- An activity restricted to nonviable cells was measured using Ala-Ala-Phe-AMC. This activity is also likely due to housekeeping aminopeptidases and is also detected with substrates using other fluorescent (rhodamine 110) or luminogenic detection groups.

CONCLUSION: Both activities were dependent on membrane integrity and were independent of other external stimuli such as proteasome inhibition or caspase induction (prior to secondary necrosis caused by these treatments).

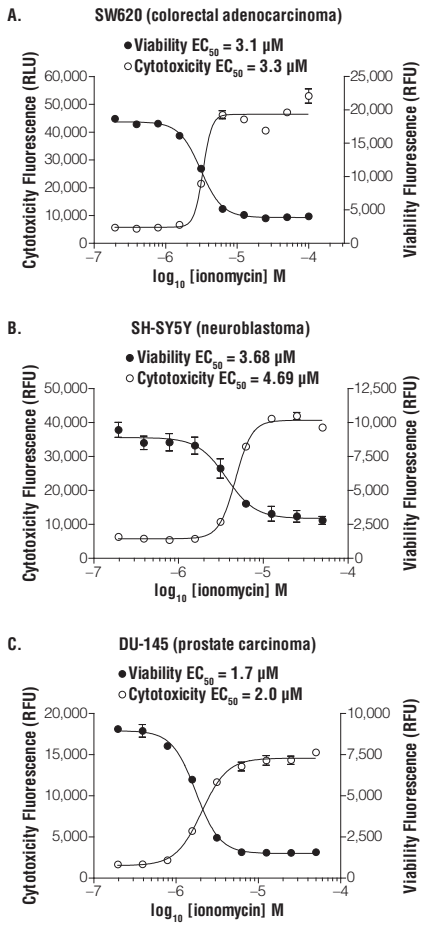
Table 1. Signals Obtained with Various Substrates During the Primary Screen.

Substrate	Viability	Cytotoxicity
Z-XXX-AMC	None*	None
Z-XXXX-AMC	None	None
Z-XXXXX-AMC	None	None
GF-AMC	+++	None
GF-AFC	+++++	None
bis-GF-R110	None	None
AAF-AMC	None	++
bis-AAF-R110	None	+++++
AAF-aminoluciferin	None	+++++
X-AMC	+	None
XX-AMC	+	None

*None denotes no statistically significant activity over control population. "+ to +++++" denote the relative strength of the response. Xs denote the number of amino acids in the substrate.

(Left) The MultiTox Assays use differential protease biomarker detection to quantify both the number of live and dead cells in a single well. By measuring both viability parameters in the same well, many sources of variability are controlled, resulting in more consistent data.

Figure 2. Potency profiles for cell viability and cytotoxicity can be easily obtained from a variety of cell types.



CHALLENGE: Find ubiquitous and highly conserved markers of cytotoxicity and viability.

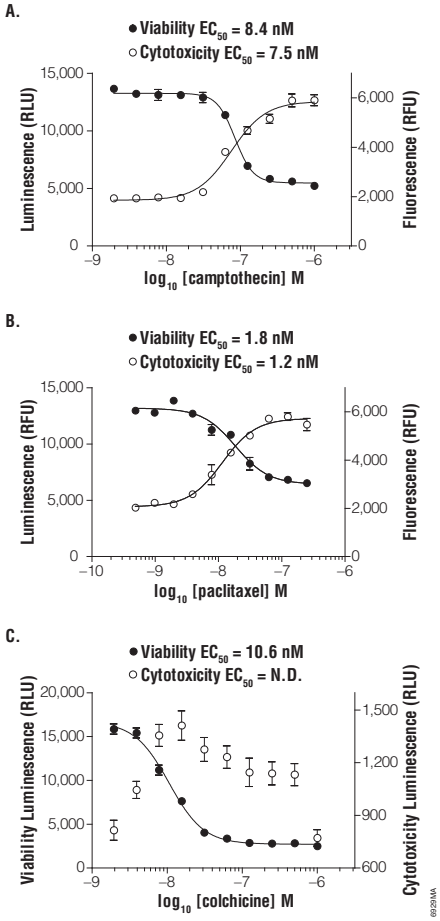
EXPERIMENT: We tested the presence and abundance of our novel proteolytic biomarkers using cell lines representing the diversity within the National Cancer Institute-60 (NCI-60) collection (Figure 2; human colon, neuron and prostate shown). This panel included cells isolated from blood, brain, colon, breast, skin, ovary, prostate, lung, and kidney tissues.

RESULTS: We observed the following:

- All cell lines tested contained "live-cell" and "dead-cell" proteases at a level useful for viability or cytotoxicity assays (less than 200 viable or nonviable cell sensitivity in limiting dilution).
- The relative abundance of the two protease-marker activities varied slightly among cell lines with a generally positive correlation, depending on cellular volume.

CONCLUSION: The proteolytic biomarkers used in these viability and cytotoxicity assays have been detected human and nonhuman mammalian cell lines (data not shown).

Figure 3. These protease-based assays yield appropriate IC_{50} values for a variety of test compounds.



CHALLENGE: Find markers that are stable enough in culture to be measured in reasonable time frames to accurately determine viability.

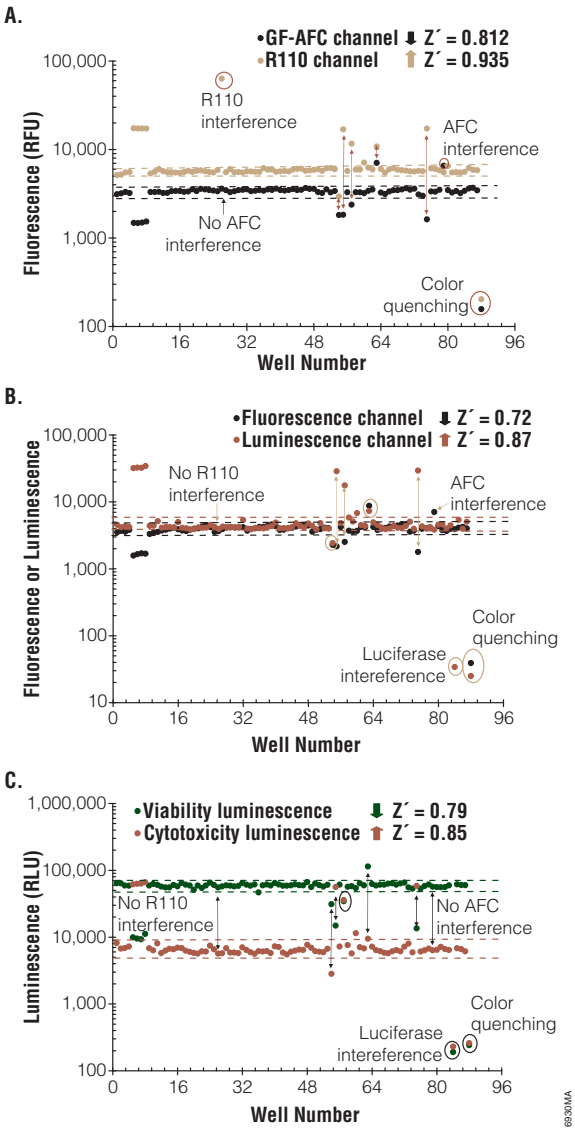
EXPERIMENT: We tested the effects of several standard cytotoxicity-inducing compounds in a broad titration series during 24- and 48-hour exposure periods.

RESULTS: We observed the following:

- The "live-cell" marker has no half-life constraints and typically delivers two asymptotes for accurate viability/potency determinations.
- The ability to detect the "dead-cell" protease marker during longer incubations is greatly dependent upon the kinetics of cell death.

CONCLUSION: Underestimation of cytotoxicity due to biomarker degradation must be considered, but kinetics and mechanism of cell death dictate the usefulness of this biomarker during extended incubations. Reduction of initial compound concentration or incubation periods often resolves this issue (Figure 3).

Figure 4. The flexible formats of these protease-based assays allow researchers to overcome most compound interference.



CHALLENGE: Develop assays that perform well in a variety of formats that limit compound interferences.

EXPERIMENT: We tested the performance of our protease biomarkers in three formats: multiplexed fluorescent (MultiTox-Fluor Assay[a]), multiplexed luminescent/fluorescent (MultiTox-Glo Assay[a,b,c]), and luminescent cytotoxicity (CytoTox-Glo™ Assay[a,b,c]). The assays were compared using a mock library containing compounds known to cause color quenching and wavelength-specific fluorescent and luminescent interference. In addition different cell numbers were added per well, with different viabilities.

RESULTS: We showed that each of the three formats performed equally for cytotoxicity but differently with respect to compound interference (Figure 4).

- All three formats demonstrated commensurate changes in viability and cytotoxicity versus control when cytotoxicity was present.
- All three chemistries "flagged" assay wells with more or less cells than control wells.
- Fluorescence interference occurred in either AFC or R110 channels, but not in both, thus "flagging" the data point. Luminescence was unaffected in both.
- Luminescence interference occurred with a luciferase inhibitor, but not with fluorescent compounds. Fluorescence was unaffected by the luciferase inhibitor.
- Color quenching agents tested affected both fluorescent and luminescent formats negatively, but dual measures allowed "flagging" of affected data points.

CONCLUSION: All three assays have relative merits with regard to their ability to "flag" potential assay interference (Table 2).

Table 2. Multiplex Viability and Cytotoxicity Screening Options.

MultiTox-Fluor Multiplex Cytotoxicity Assay	MultiTox-Glo Multiplex Cytotoxicity Assay	CytoTox-Glo™ Cytotoxicity Assay
Single-addition reagent	Two-step reagent addition	Two-step reagent addition
Nonlytic	Nonlytic	Lytic second step
96-, 384-, 1536-well plate formats	96-, 384-, 1536-well plate formats	96-, 384-, 1536-well plate formats
Avoids known luminescence inhibitors	Good "hedge" for unknown libraries	Avoids fluorescence interference
Can be multiplexed with other luminescence assays	Enhanced sensitivity from luminescence format	Enhanced sensitivity from luminescence format
Flags problem data points	Flags problem data points	Flags problem data points

Multiplex Cytotoxicity Assays

Summary

Several sensitive and robust protease biomaker assay chemistry options allow you to choose the best assay for your chemical library, treatment regimens, and desired endpoint. The markers are constitutive and conserved and may be used to distinguish between changes in viability and cytotoxicity. Ultimately, flexibility within detection platforms allows researchers to balance multiplex features with throughput and improve data quality.

Qualify for a FREE SAMPLE of the MultiTox-Fluor Assay at: www.promega.com/multitoxfluor_cn019/

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References

1. Niles, A. *et al.* (2007) *Anal. Biochem.* **366**, 197–206.
2. Niles, A. *et al.* (2006) *Cell Notes* **15**, 11–5.
3. Niles, A. *et al.* (2006) *Cell Notes* **16**, 12–5.
4. Niles, A. *et al.* (2007) *Cell Notes* **18**, 15–20.

Ordering Information

Product	Size	Cat.#
MultiTox-Fluor Multiplex Cytotoxicity Assay	10 ml	G9200
CytoTox-Fluor™ Cytotoxicity Assay	10 ml	G9260
CytoTox-Glo™ Cytotoxicity Assay	10 ml	G9290
MultiTox-Glo Multiplex Cytotoxicity Assay	10 ml	G9270
CellTiter-Fluor™ Cell Viability Assay	10 ml	G6080

For laboratory use. For invitro use only. Additional Sizes Available.

[a]Patent Pending.

[b]U.S. Pat. Nos. 6,602,677 and 7,241,584, Australian Pat. No. 754312 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.

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