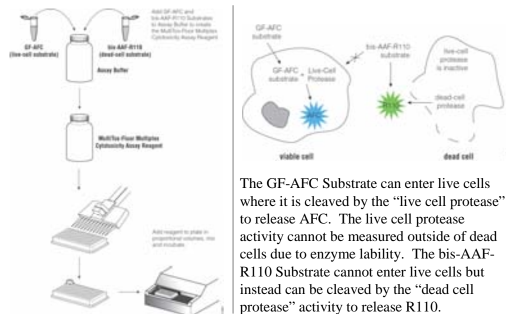
A Multiplex Viability and Cytotoxicity Assay for Improving Screening Data

Andrew Niles*, Tracy Worzella*, Richard Moravec*, William Daily#, Michael Scurria#, Laurent Bernad#, Brian McNamara*, Kay Rashka*, Deborah Lange*, and Terry Riss*.
*Promega Corporation, Madison, WI 53711 and #Promega Biosciences, San Luis Obispo, CA 93401

Abstract

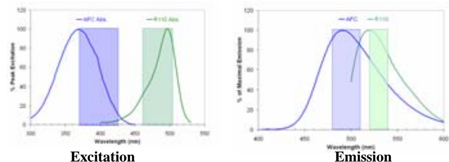
We have developed a homogeneous assay chemistry that simultaneously measures the relative number of live and dead cells in culture by detecting changes in cell membrane integrity. This single addition assay measures two constitutive proteolytic activities; one is a marker of viability, and the other a marker of cytotoxicity. Together, these measures provide an inversely complimentary viability profile for each assay well. The resulting ratiometric assay data can also be used to improve additional multiplex endpoint data quality by response normalization, by increasing content, and by mitigating false negative or positive determinations. Here we detail advantages of the assay in simple cytotoxicity screening and in multiplexed cell-based models of necrosis and apoptosis. The functionality, sensitivity, and utility of the assay will be described using high density, multi-well formats with conventional fluid handling robotics and standard fluorometers or luminometers.

Reagent Preparation and Assay Principle



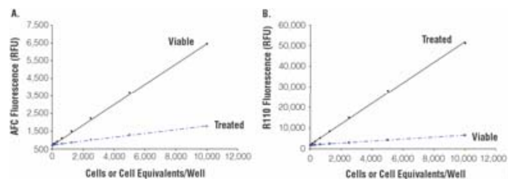
The GF-AFC Substrate can enter live cells where it is cleaved by the "live cell protease" to release AFC. The live cell protease activity cannot be measured outside of dead cells due to enzyme lability. The bis-AAF-R110 Substrate cannot enter live cells but instead can be cleaved by the "dead cell protease" activity to release R110.

Multiplex Signal Separation



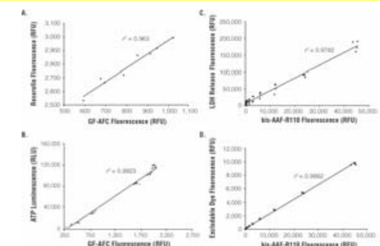
Fluorogenic dyes exhibit distinct absorption (excitation) and emission profiles when a laser energy source is applied. In multiplexed formats, there is often modest overlap in these profiles beyond their optimal peaks. Most fluorometers (or multi-mode instruments) contain optical band-pass filters that restrict the spectral wavelengths used to excite a fluorophore and collect the resulting photon emission. Therefore, the signal generated from live cells can be optically separated from the dead cells by measuring the signals in different channels.

Differential Viability and Cytotoxicity Measures



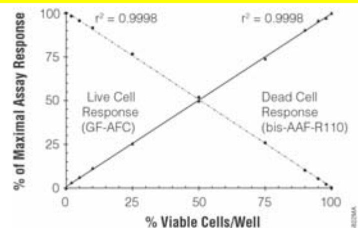
The signals derived from viable cells or cells subjected to cytotoxic treatment are proportional to cell number. Panel A shows the signal associated with viable cells versus dead cells at $400_{Ex}/505_{Em}$. Panel B shows the signal associated with dead cells versus viable cells at $485_{Ex}/520_{Em}$.

Assay Correlation with Existing Methods



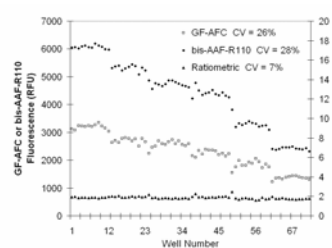
The distinct proteolytic activities measured in the assay correlate well with established methods for viability and cytotoxicity. Panel A. GF-AFC versus CellTiter-Blue®. Panel B. GF-AFC versus CellTiter-Glo®. Panel C. Bis-AAF-R110 versus CytoTox-ONE™. Panel D. Bis-AAF-R110 versus ethidium homodimer.

Inversely Correlated, Ratiometric Measures



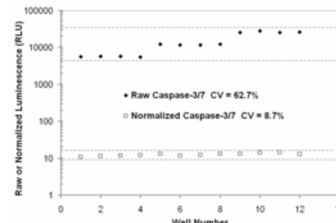
When viability is high, the GF-AFC signal will be high and bis-AAF-R110 signal will be low (compared to control). When viability is low, the GF-AFC signal will be low and the bis-AAF-R110 signal will be high. Viability changes as small as 2% (200 Jurkat cells, above) can be detected in a population of 10,000 cells per well.

Improving Assay Precision by Ratiometric Means



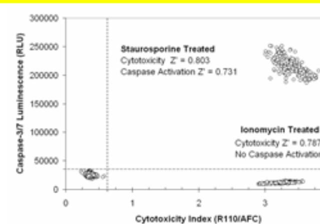
Single parameter assay responses vary greatly with cell number. The values derived from 12,500; 11,000; 10,000; 9,000; 7,500 and 5,000 cells per well at 50% viability are shown as single parameter and ratiometric values. The quotient of the R110 and AFC values (ratiometric value) resolves much of the observed variation due to cell number.

Normalizing Primary Response Data



Defining cell number prior to determining other primary response data (caspase activation, genetic reporter activity, etc) can normalize the data. U266 cells were seeded at 5000, 10000, and 20000 cells/well and treated with staurosporine. Viable cell number was determined, then caspase activation measured in a luminescent multiplex. Data are plotted as raw and normalized values (caspase response/viability response).

Increasing Content by Downstream Multiplex



Key

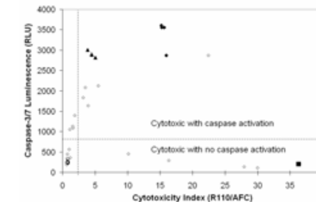
"Cytotoxicity Index" is raw R110 values divided by raw AFC values. Larger values indicate more cytotoxicity.

Cytotoxic Z' values generated from the average of each treatment well replicates versus untreated control wells.

Caspase activation Z' generated from the average of staurosporine treated wells versus untreated control wells

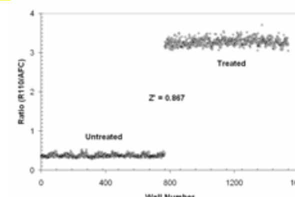
Jurkat cells were seeded at 5000 cells/well in a 384 well plate and subjected to either ionomycin or staurosporine treatment. Viability and cytoxicity were determined, then caspase activation measured with a multiplexed luminescent assay. Addition of the caspase activation parameter can separate differential modes of cytotoxicity (apoptotic versus necrotic profiles).

Cytotoxicity and Apoptosis Screening



Jurkat cells (5000/well) were exposed to compounds from LOPAC plate 4, at 10µM for 8hrs. Detergent (square), Anti-Fas mAb (diamond) or staurosporine (triangle) served as necrosis or apoptosis controls, respectively. Several compounds from the LOPAC screen induced cytotoxicity by apoptosis or necrosis.

High Z' in a 1536 Well Format



Jurkat cells were adjusted to 625,000 cells/ml then divided into two fractions. One fraction was treated with detergent to simulate cytotoxicity (treated) the other left untreated. 4µl of treated and untreated cells were added to a 1536 well plate. 4µl of the Reagent was added and viability and cytotoxicity determined after 30 minutes of incubation. Z' was calculated as a ratio of R110 to AFC for each well instead of the conventional single parameter averages of untreated versus treated measurements.

Summary

- We have developed a new homogeneous, multiplex assay chemistry for simultaneously determining the relative number of live and dead cells in an assay well.
- The assay uses two proteolytic substrates which yield spectrally distinct fluorophore products when in the presence of viable and cytotoxic cells.
- The data from the assay correlate well with other measures of cytotoxicity.
- The resulting data are inversely complimentary and can be used to increase assay precision by ratiometric normalization.
- The assay can be multiplexed with other spectrally distinct readouts such as a luminescent caspase assay to increase content or determine mode of cytotoxicity.
- The assay demonstrates high sensitivity and Z' values in high density cell-based formats (384 and 1536).

Contact: Andrew Niles (608) 274-4330 extension 1447 or andrew.niles@promega.com