

Multiplexing Cell-Based Assays

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Abstract

Multiplex recording of more than one indicator from the same sample increases the efficiency of assay development and screening. Recent advances in cell-based assay chemistries have made it possible to record multiplex data in a high throughput format using standard plate readers without the need to perform microscopic imaging of individual cells. Identification of novel markers of cell viability and development of detection chemistries that do not kill cells have expanded the possible combinations for multiplexing cell-based assays in microwell plates. We have developed methods to combine various luminescent and fluorescent cell-based assays in a homogeneous format. We have recorded luciferase reporter gene assays in real time in living cells followed by measuring cell viability or caspase activity. Measuring the number of viable cells at the end of a treatment period is useful to distinguish between a specific down regulation of a reporter gene or non-specific cytotoxicity. Multiplexed cell viability data also can serve as an internal control to correct for variability in seeding density and differential growth of cells resulting in improvements in the reliability of data. We have also developed methods for simultaneous detection of viable and dead cells in the same sample and have demonstrated multiplexing of homogeneous fluorescent cell viability and luminescent caspase assays in the same well. We will present the results of ongoing efforts to investigate the compatibility of different assay chemistries and detection methods for developing multiplexed homogeneous cell-based assays with sensitivity sufficient for microwell assays.

Introduction

There are several possibilities to combine homogeneous cell-based assays in a single well and record multiplex data using standard plate readers in a high throughput format.

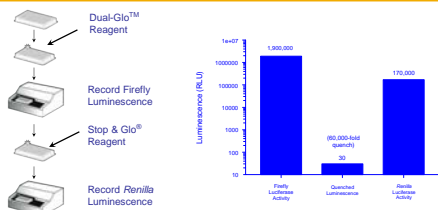
The basic requirements for multiplexing include:

- the detection signals must be distinguishable from each other and
- the assay chemistries must be compatible or separable from each other.

Assays can be combined for multiplexing in a...

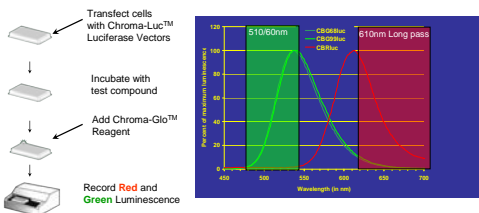
- **Simultaneous** format where assays are done at the same time,
- **Sequential** format where the 1st assay is completed before addition of the 2nd assay reagent, or a
- **Split** format where the original sample is divided into different compartments or wells to separate otherwise incompatible assay chemistries.

Sequential Dual-Glo™ Luminescent Reporter Assays



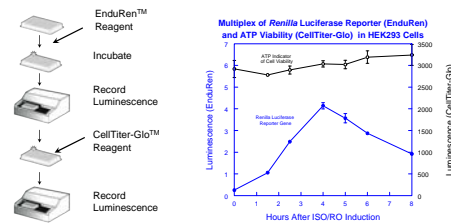
Homogeneous assay format for a dual luciferase reporter gene assay. Cells are prepared to express firefly and *Renilla* luciferase reporters linked to genes of interest. Sequential addition of two reagents allows recording of firefly luciferase first. The 2nd reagent quenches firefly luciferase 60,000-fold and provides the substrate for *Renilla* luciferase thus allowing both luminescent reporters to be recorded in a sequential fashion from the same well.

Simultaneous Luminescent Reporter Gene Assays



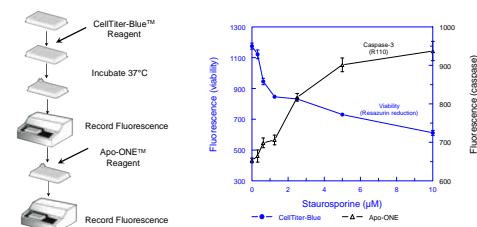
Flow diagram shows the homogeneous format for a dual color reporter gene assay. Cells are prepared to contain the Chroma-Luc Vectors expressing 2 different click beetle luciferases (gene of interest and control for viability). Chroma-Glo Reagent containing luciferase substrate is added in a single step and incubated. Luminescence is recorded using two different filter sets to distinguish between expression levels of the red and green luciferase. Both reporters are recorded simultaneously with different filter sets, from the same well.

Multiplex Reporter Gene & Cell Viability Assays



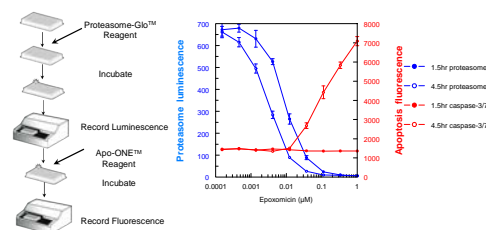
Sequential homogeneous assay protocol to measure *Renilla* luciferase reporter gene activity in live cells, followed by measuring cell viability as a control. Reporter gene activity in living cells increased over time, but cell number (ATP content) remained constant. The multiplex data confirm the increase in luminescence was due to specific up-regulation of luciferase expression rather than an increase in viable cell number.

Cell Viability and Apoptosis



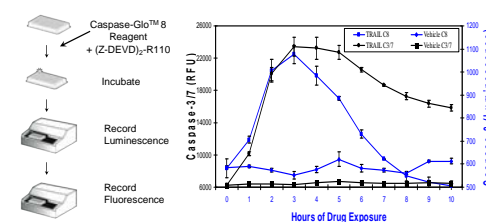
Sequential homogeneous addition of CellTiter-Blue® Reagent to measure cell viability (resazurin reduction to fluorescent resorufin), followed by addition of Apo-ONE® Reagent to measure apoptosis (caspase-3) in the same well. Emission of R110 and resorufin fluorescence are separated enough to allow both endpoints to be measured in single well. The multiplex data show a decrease in the marker for cell viability and an increase in the marker for apoptosis.

Cell-Based Proteasome and Apoptosis Assays



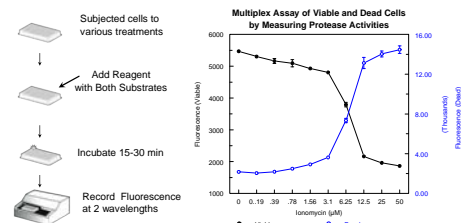
Sequential homogeneous addition of Proteasome-Glo™ Reagent to measure proteasome activity in cells using luminescence, followed by addition of Apo-ONE® Reagent to measure apoptosis (caspase-3) in the same well using fluorescence. Epoxomicin inhibits the proteasome at 1 hour without induction of caspase activity. At 4.5 hours, epoxomicin above 0.04µM induces apoptosis.

Simultaneous Protease Assays: Luminescent Caspase-8 & Fluorescent Caspase-3



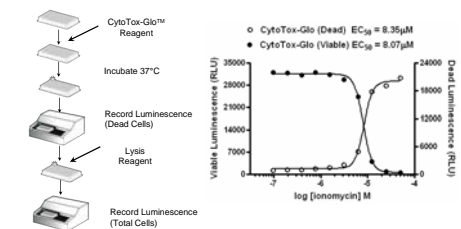
Homogeneous dual assay protocol to measure caspase-8 using a luminescent Caspase-Glo™ 8 substrate [Z-LETD-aminoluciferin] and caspase-3 using a fluorescent Apo-ONE® substrate [(Z-DEVD)-R110] simultaneously in the same sample wells. The luminescent protease assay format provides the sensitivity to enable measurement of caspase-8 from the number of cells typically used in 96 well format. As expected, the activity of the upstream signaling caspase (caspase-8) diminishes before the executioner caspase (caspase-3).

Viable Cells and Dead Cells Simultaneously



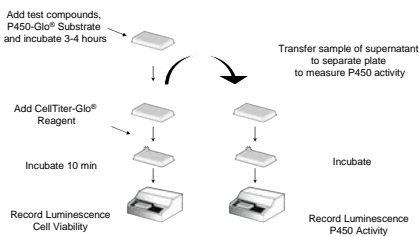
Simultaneous homogeneous format to measure viable and dead cells in the same sample. A cell permeable fluorogenic substrate (GF-AFC) is selectively cleaved by protease activity restricted to viable cells. A second impermeable fluorogenic substrate (bis-AAF-R110) is used to measure a protease activity released from dead cells.

Dual Luminescent Cytotoxicity and Viability Assay



CytoTox-Glo™ Reagent containing a luminescent dead cell protease substrate (AAF-aminoluciferin) measures the number of dead cells. Subsequent addition of lysis reagent measures the same protease activity from all cells present (both viable and non-viable populations). Data points from the two measures can be used in a ratiometric manner to normalize for variation introduced through cell clumping or pipetting errors.

Split format to Multiplex P450 Activity and Cell Viability



Separation of culture medium into a separate plate after incubation with cell permeable luminescent P450 substrate can enable measurement of cell viability from the original samples using CellTiter-Glo® luminescent ATP assay. Cell viability values can be used to normalize for potential cytotoxic effects

Summary

- Several different cell-based assay combinations are possible that can be recorded using standard plate readers.
- You do not need a HCS imaging instrument to be able to multiplex cell-based assays.
- Multiplexing can save time and be more cost efficient than parallel assays on different samples.
- Measuring 2 or more parameters from the same sample provides more consistency than using parallel samples.
- Multiplexed assays can be used for data normalization to correct for cytotoxicity or pipetting errors.
- Knowledge of the assay chemistries will help determine if assay combinations are possible.
- For additional technical information for all of the individual assays, see <http://www.promega.com>