

OVERVIEW

To meet demands for measuring multiple parameters from a single sample, we demonstrate the utility of Promega cell-based assays, including reporter assays, in multiplex experiments. Here we provide proof-of-principle data by combining Promega's cell-based screening assays in a multiplexed format with a variety of high-throughput liquid handling and detection instrumentation. The data show the ability to combine assay chemistries with laboratory instrumentation, in a high-throughput setting, in order to obtain more information from one sample well.

INTRODUCTION

Today's high-throughput screening facilities face increasing demands to generate more information from their existing compound libraries. This can be accomplished by two separate means. The first is to run additional assays in a series. While this may produce the desired data, it also lengthens drug development time, as more assays need to be set up and run by screening groups. Furthermore, this format drives up costs because of additional compound, labware and tissue culture component consumption. A second and more appealing choice is to run these same assays in a multiplex format in which multiple chemistries are used to evaluate multiple parameters of a single sample. This configuration helps to speed up the discovery process because more than one piece of information can be discerned from each screening run. A multiplexed format also decreases costs and variability because different assays are performed using the same plate of cells.

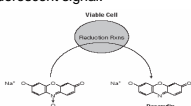
High-precision liquid handling and detection instrumentation can also bring assay multiplexing closer to the high-throughput screening stages of drug development. Here we used the Tecan Freedom EVO® platform to dispense cells, treatment, and assay reagents to plates in 96-, and 384-well format. The Deerac Fluidics™ Equator™ HTS was used to perform similar dispensing in Low Volume 384 (LV384), and 1536-well format. Tecan's GENios Pro was used to detect fluorescent and luminescent signals in 96-, and 384-well format, while the BMG PHERAstar detected signals in LV384, and 1536-well format. (96- and LV384-well data not shown here.)

The homogeneous "add, mix, measure" format of these assays makes them simple to use and automate. The luminescent or fluorescent readouts from the assays, combined with compatible instrumentation, makes these assays highly amenable to high-throughput multiplexing.

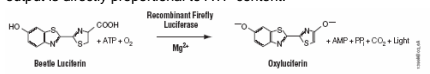
METHODS

Different reagent chemistries were selected to demonstrate the ability to multiplex assays, with each assay measuring a different cellular process or event. The following assays were used to demonstrate the multiplexing applications represented here.

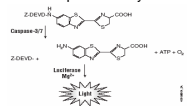
CellTiter-Blue®: a non-lytic cell viability assay that is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Viable cells retain the ability to reduce resazurin into resorufin. Nonviable cells rapidly lose metabolic capacity, do not reduce the indicator dye, and thus do not generate a fluorescent signal.



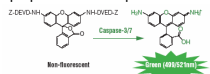
CellTiter-Glo®: a luminescent, lytic assay that determines the number of metabolically active, viable cells based on the quantitation of ATP present. In the presence of ATP and oxygen, luciferase acts on the luciferin substrate and produces light. Light output is directly proportional to ATP content.



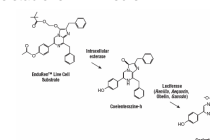
Caspase-Glo® 3/7: a luminescent, lytic assay that measures caspase-3 and -7 activity in cells undergoing apoptosis. In the presence of caspases -3 and -7, a tetrapeptide Z-DEVD substrate is cleaved, producing luciferin which further reacts with luciferase to generate light. Light output is directly proportional to caspase activity.



Apo-ONE®: a fluorescent, lytic assay that measures caspase-3 and -7 activity in cells undergoing apoptosis. In the presence of caspase -3 and -7, a tetrapeptide Z-DEVD-R110 substrate is cleaved, and the Rhodamine 110 leaving group becomes intensely fluorescent. Fluorescence is proportional to caspase -3/7 activity.



EnduRen™ Live Cell Substrate: a protected coelenterazine substrate that is designed to generate Renilla luciferase luminescence from living cells. Once inside the cell, the protected EnduRen™ substrate is cleaved by intracellular esterases, generating coelenterazine which reacts with Renilla luciferase to produce light. Peak luminescence is achieved within 1.5 hours of substrate addition to cell culture wells, and luminescence is stable for > 24 hours.



ViviRen™ Live Cell Substrate: a protected coelenterazine substrate that is designed to generate Renilla luciferase luminescence from living cells. Once inside the cell, the protected ViviRen™ substrate is cleaved by intracellular esterases, generating coelenterazine which reacts with Renilla luciferase to produce light. ViviRen™ Substrate will generate nearly maximal luminescence approximately 2 minutes after addition and the luminescent signal will then decrease in intensity, with a half-life of 8-15 minutes.

INSTRUMENTATION

Tecan Freedom EVO®: For these multiplex applications, the Tecan Freedom EVO® liquid handler was used for dispensing cells, compound treatments, and assay reagents into 96 and 384-well tissue culture plates. The Freedom EVO® configuration included an 8-channel LiHa pipetting system, RoMa gripper tool, and an H+P Labortechnik VARIOMAG TELESHAKE.



Tecan GENios Pro™: An off-line GENios Pro™ plate reader was used to measure both luminescent and fluorescent signal outputs from the various multiplexed assay combinations presented here. The GENios Pro™ can read both 96 and 384-well plates for a variety of readout modes including, but not limited to, fluorescence intensity and luminescence used here. The GENios Pro™ was used for top-well reading in endpoint mode.

INSTRUMENTATION

The Deerac Fluidics™ Equator™ HTS Eight Tip Pipetting System was used to dispense cells, compound treatments, and assay reagent in LV384, and 1536-well format. The Equator™ HTS dispenses volumes ranging from 200ul down to 50nl in 96, 384, and 1536-well plate formats using on-the-fly pipetting technology. The system is designed for high to ultra-high-throughput applications. The system channels can all individually aspirate and dispense varying volumes with high accuracy and reproducibility. The Equator™ HTS system can be configured according to the specific requirements of the customers assay with interchangeable small or large volume capacity reservoirs, stirring devices and multiple wash stations.

Reagents were placed into a 96-well microtiter plate, which was then placed onto the deck of the instrument. A second low-volume 384- or 1536-well plate was then added to the deck to perform the assay.



RESULTS – Cell Viability Multiplexed with Apoptosis

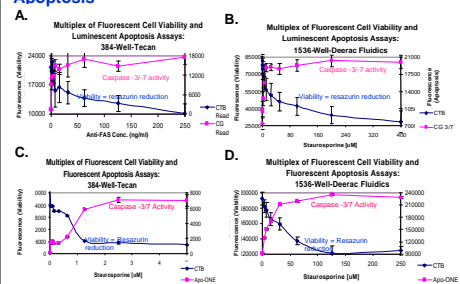


Figure 1: Results show that with increasing concentration of test compound, a decrease is seen in cell viability, coupled with an increase in caspase 3/7 activity. This suggests that cells are undergoing apoptosis, as opposed to necrosis. **Panels A and B.** Fluorescent Cell Viability with Luminescent Apoptosis Assay. **Panels C and D.** Fluorescent Cell Viability with Fluorescent Apoptosis Assay.

ASSAY SETUP

- Cell Plating
 - 384-Well: Jurkat Cells / 2x10⁴ Cells/Well (CellTiter Blue® w/Caspase-Glo® 3/7)
 - 384-Well: CHO Cells / 2x10⁴ Cells/Well (CellTiter Blue® w/Apo-ONE®)
 - 1536-Well: HEK293 Cells / 4x10³ Cells/Well
- Apoptosis Induction
 - Jurkat Cells: anti-FAS antibody Treatment / 4 Hr. Incubation at 37°C/5%CO₂
 - CHO Cells: Staurosporine Treatment / 16 Hr. Incubation at 37°C/5%CO₂
 - HEK293 Cells: Staurosporine Treatment / 16 Hr. Incubation at 37°C/10%CO₂
- CellTiter-Blue® reagent added 2 Hrs. prior to end of treatment
 - CellTiter-Blue® conc. 1:4 Dil. w/PBS when combined with Caspase-Glo® 3/7
 - CellTiter-Blue® conc. 1X when combined with Apo-ONE®
- Fluorescence signal recorded at 560(20)ex/590(10)em
- Apo-ONE® or Caspase-Glo® 3/7 reagent added and plates incubated at RT for 30 minutes or 60 minutes respectively
- Luminescent signal recorded

Results show the ability to assay the same experimental wells with a fluorescent viability assay combined with either a fluorescent or luminescent apoptosis assay in multiple well formats.

RESULTS – Cell Viability Multiplexed with Reporter Gene Activity

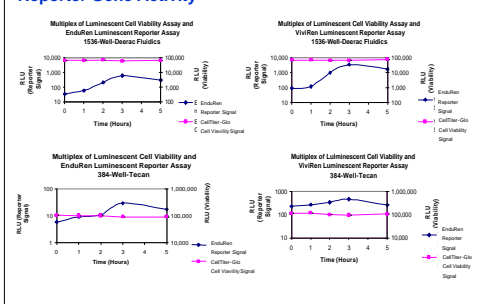


Figure 2. Measurement of CRE induction with either EnduRen™ or ViviRen™ Substrate with subsequent monitoring of cell viability within the same well. HEK 293 cells stably transfected with an inducible CRE/CL1 hPEST Renilla luciferase construct (2) were plated at 96-, ST384-, LV384-, and 1536-well format and allowed to attach for 10 hours at 37°C/5% CO₂. EnduRen™ Substrate was mixed with the cells and media at a final concentration of 60µM two hours prior to induction. Ten micromolar isoproterenol was added to all test plates to induce the CRE. ViviRen™ Substrate was added by the liquid handling workstation to the appropriate test plates at final concentration of 60µM. Induction was monitored at 0, 1-, 2-, 3- and 5-hour time points. At each time point, Renilla luminescence was recorded, followed by addition of the CellTiter-Glo® Reagent by the liquid handling workstation. Luminescence was recorded a second time to measure ATP content and cell number. Graphs above were generated in the following formats using the instruments indicated: **Panels A and B.** 1536-well format, Equator™ HTS workstation and PHERAstar detection instrument. **Panels C and D.** ST384-well format, Freedom EVO™ Workstation and GENios-Pro detection instrument.

Results show the ability to track the response of a reporter gene over time by measuring luminescent Renilla reporter activity followed by assessment of cell viability in the same experimental well using a luminescent assay. For this application, the Renilla reporter expression was optimal at 3 hours of treatment with 10µM isoproterenol in all assay formats. The results also show the increased luminescence generated by the ViviRen™ Substrate over the EnduRen™ Substrate. This can be of great benefit when volume as well as signal is decreased in a miniaturized assay.

CONCLUSIONS

- Each of the experiments performed here demonstrates the ability to perform cell-based assays, including reporter assays, in a multiplex format on a wide variety of robotic platforms.
- Promega's cell-based assays, represented here, have compatible reagent chemistries that can be used when multiplexing is desirable.
- Multiplexed assays can be performed with laboratory liquid handlers and plate readers, both in standard and miniaturized formats.
- The Freedom EVO® is a flexible, high-throughput platform for performing multiplexed assays in 96 and 384-well formats.
- The Deerac Fluidics™ Equator HTS platform is a flexible and easy to use non-contact nanoliter pipetting system capable of dispensing cells, compound, and reagents for high-throughput, low volume screening assays.
- The GENios Pro™ plate reader is an excellent option for detecting both luminescent and fluorescent output signals, in 96- and 384-well format, from the same assay well.