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OVERVIEW

Here we demonstrate the ability to multiplex select Promega cell-based assays using a standard laboratory liquid handler and plate reader. The Tecan Freedom EVO liquid handler was used for delivering cell-based assay reagents to 96 and 384-well tissue culture plates. The GENios Pro reader was used to measure luminescent and fluorescent output signals from the same assay plate. The data show the ability to combine assay chemistries with laboratory instrumentation in order to obtain more information from one sample well.

INTRODUCTION

Using cells in the drug discovery process enables the researcher to screen for drug effects within the context of the cellular biological environment. It is not uncommon for one cellular event to trigger another, and capturing information about immediate and downstream effects can be critical in developing an understanding of the different pathways activated during drug treatment. Assay multiplexing is one way in which the researcher can gain more information from their treatment while at the same time save time, money and gain more reproducible results by using the same treated sample for analysis. High-precision liquid handling and detection instrumentation combined with robust, compatible reagent chemistries, can bring assay multiplexing closer to the high-throughput screening stages of drug development.

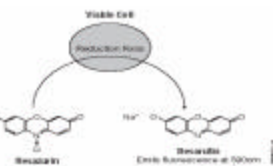
Promega's suite of cell-based assays allow the researcher to analyze a variety of cellular events including apoptosis, cytotoxicity, cell viability, and protein expression. The homogeneous "add, mix, measure" format of these assays makes them simple to use and automate. The luminescent or fluorescent readouts from the assays, combined with compatible reagent chemistries, makes these assays highly amenable to multiplexing. Here we demonstrate the use of Promega's CellTiter-Blue®, CellTiter-Glo®, Caspase-Glo® 3/7, Apo-ONE®, EnduRen™ and ViviRen™ Live Cell Substrates in multiplexed combinations performed within the same assay well.

Automation plays an important role in the screening process. Here we demonstrate the use of the Tecan Freedom EVO and GENios Pro reader to dispense multiplexed assay reagents and record signal outputs in different modes, respectively.

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.

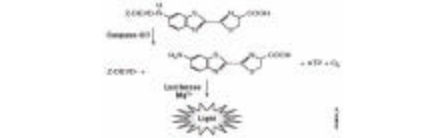
CellTiter-Blue®: a non-lytic cell viability assay that is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). Viable cells retain the ability to reduce resazurin into resorufin. Nonviable cells rapidly lose metabolic capacity, do not reduce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



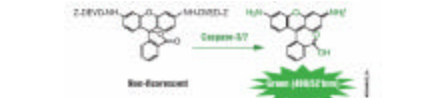
CellTiter-Glo®: a luminescent, lytic assay that determines the number of metabolically active, viable cells based on the quantitation of ATP present. In the presence of ATP and oxygen, luciferase acts on the luciferin substrate and produces light. Light output is directly proportional to ATP content.



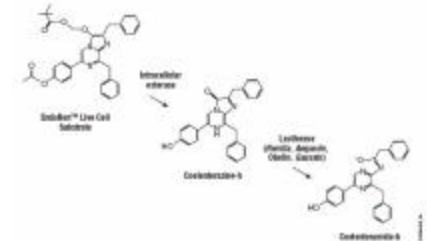
Caspase-Glo® 3/7: a luminescent, lytic assay that measures caspase-3 and -7 activity in cells undergoing apoptosis. In the presence of caspases -3 and -7, a tetrapeptide DEVD substrate is cleaved, producing luciferin which further reacts with luciferase to generate light. Light output is directly proportional 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 ZDEVD-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 cell assay reagents into 96 and 384-well tissue culture plates. The Freedom EVO configuration included an 8channel 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.

RESULTS

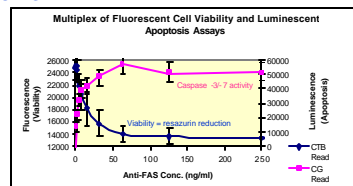


Figure 1. CellTiter-Blue® / Caspase-Glo® 3/7 Multiplex. Jurkat cells were manually plated at a density of 5X10⁴ cells per well in a 96-well white tissue culture plate (Costar #3610). To induce apoptosis, anti-FAS antibody was manually added at varying concentrations, and the assay plate was incubated at 37°C for 2 hours. The Freedom EVO was used to dispense CellTiter-Blue® reagent to each well of the assay plate, and the plate was further incubated for 2 hours at 37°C. The GENios Pro plate reader was used to record fluorescence at 560(20)_{ex}/590(10)_{em}. The Freedom EVO was then used to add Caspase-Glo® 3/7 reagent to the plate. Followed by a one-minute shake, the plate was incubated for 30 minutes, and luminescence was recorded with the GENios Pro.

Results show the ability to assay the same experimental wells with both a luminescent viability assay and a fluorescent apoptosis assay.

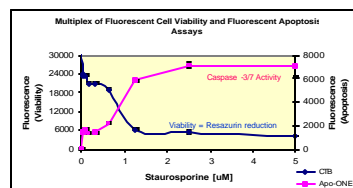


Figure 2. CellTiter-Blue® / Apo-ONE® Multiplex. CHO cells were plated manually at 2 x 10⁴ cells per well in a Costar 384 well tissue culture plate (Costar #3707), allowed to attach for 10 hours, then manually treated with varying doses of staurosporine for 16 hours at 37°C/ 5%CO₂. The Freedom EVO was used to add CellTiter-Blue® reagent, plates were incubated at 37°C/ 5%CO₂ for 2 hours and fluorescence units were recorded with the GENios Pro (560(20)_{ex}/590(10)_{em}). The Freedom EVO was then used to add Apo-ONE® reagent, the plate was incubated at room temperature for one hour, and fluorescence was recorded with the GENios Pro (485_{ex}/520_{em}). Results show the ability to assay the same experimental wells with a fluorescent viability assay and a fluorescent apoptosis assay using a 384-well plate.

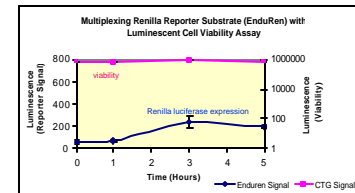


Figure 3. EnduRen™ Live Cell Substrate / CellTiter-Glo® Multiplex. HEK 293 cells stably transfected with an inducible CRE/ CL1pEST Renilla luciferase construct were manually plated at 2 x 10⁴ cells per well in three 96-well white tissue culture plates (Costar#3610) and allowed to attach overnight at 37°C/5%CO₂. At the time of plating, EnduRen™ substrate was added to the tissue culture media. The next day, 6µM of isoproterenol was added to each tissue culture plate, the plates were incubated at 37°C/ 5% CO₂ and removed at 1, 3, and 5-hour time points. At each time point, Renilla luminescence was recorded with the GENios Pro, followed by addition of CellTiter-Glo® reagent with the Freedom EVO. Luminescence was recorded a second time to measure ATP content and cell number.

Results show the ability to measure luminescent Renilla reporter activity and subsequent luminescence for cell viability in the same experimental well. For this application, the Renilla reporter expression was optimal at 3 hours of treatment with the isoproterenol.

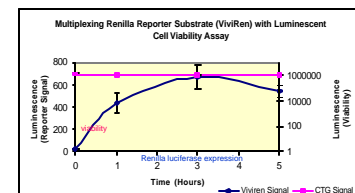


Figure 4. ViviRen™ Live Cell Substrate / CellTiter-Glo® Multiplex. The experiment described for Figure 3 was repeated with stably transfected HEK 293 cells and 6µM of isoproterenol, only this time the ViviRen™ substrate was used in place of the EnduRen™. Since the ViviRen™ signal has a shorter half-life than EnduRen™, the substrate was added with the Biomek® FX before each time point, the plates were incubated for 2 minutes at room temperature, and the Renilla luminescence was recorded within 15-minutes of the substrate addition. CellTiter-Glo® reagent was then added with the Biomek® FX as described in Figure 3. Using the ViviRen™ substrate resulted in the generation of a brighter luminescent signal as compared to that from the EnduRen™. The ViviRen™ substrate is an option for lower expression Renilla luciferase constructs such that light units are detectable above assay background.

CONCLUSIONS

- Several different cell-based assay combinations are possible for multiplexing.
- Promega's cell-based assays represented here have compatible reagent chemistries that allow for multiplexing of two different assays in one sample well.
- Multiplexed assays can be performed with laboratory liquid handlers and standard plate readers.
- The Freedom EVO is a flexible, high-throughput platform for performing multiplexed assays in 96 and 384-well formats.
- The GENios Pro plate reader is an excellent option for detecting both luminescent and fluorescent output signals from the same assay well.