PERFORM MULTIPLEXED CELL-BASED ASSAYS ON AUTOMATED PLATFORMS

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To meet demands for measuring multiple parameters from a single sample, we present data demonstrating the utility of Promega cell-based assays, including reporter assays, in multiplex experiments. The multiplex experiments were run on a variety of automated platforms using an assortment of detection instruments.

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.

The single-reagent additions, extended signal half-lives and sensitivity of each of these chemistry combinations make them ideal for automated high-throughput or ultrahigh-throughput liquid handling and detection.

While performing assays in a multiplexed format may be more appealing to high-throughput screening facilities, there are still barriers that prevent laboratories from changing to this configuration. Short deadlines and increased pressure to identify lead compounds have created a situation where many groups do not have the time or personnel available to develop new assays. Without supporting data to show that unique assay combinations will work in their labs, researchers will continue to use existing designs, even if those original assay designs are not advantageous. Here we provide proof-of-principle data showing that multiplexed assay configurations previously tested in a manual format (1) can also be performed successfully in an automated high-throughput or ultrahigh-throughput setting.

We multiplexed a nonlytic fluorescent cell viability assay, the CellTiter-Blue® Cell Viability Assay, with either lytic fluorescent or luminescent apoptosis assays, the Apo-ONE® Homogeneous Caspase-3/7 Assay or Caspase-Glo® 3/7 Assay, respectively. Experiments were run using both suspension and adherent cell lines. We also multiplexed reporter assays using two live-cell reporter substrates, EnduRen™ Live Cell Substrate and ViviRen™ Live Cell Substrate, with the lytic CellTiter-Glo® Luminescent Cell Viability Assay. Reporter-gene induction was assayed in these multiplex experiments. All chemistry combinations were tested in 96-, standard 384- (ST384), low-volume 384- (LV384), and 1536-well formats.

METHODS

Automated Liquid Handling

Multiplexed assays were tested in 96- and ST384-well formats using either the Tecan Freedom EVO™ 200 or the Beckman Coulter Biomek® FX workstation (Table 1). Tests were also run in automated LV384- and 1536-well formats using the Deerac Fluidics™ Equator™ HTS low-volume liquid dispenser (Table 1). The Freedom EVO™ 200 used for these experiments had an expandable 8-tip liquid handler with a separate plate gripper. The Biomek® FX had a 96-tip head with an integrated plate gripper. Both automated platforms contained an orbital plate shaker for mixing cells and reagents. These two automated platforms represent systems commonly found in screening facilities running 96- and 384-well format assays. The Equator™ HTS workstation that was used for these experiments is a noncontact low-volume 8-tip liquid dispenser. No plate shaker was required with this robotic platform, as the combined dispense force and time was sufficient to mix cells and reagents. The noncontact dispensing ability of the Equator™ HTS workstation allows accurate pipetting into high-density LV384- and 1536-well plates in a short period of time with no disposable tip usage, required features for ultraHTS laboratories.

High-Throughput Detection Systems

The Tecan GENios-Pro detection instrument was used to analyze assay plates run on the Freedom EVO™ workstation, while the Beckman Coulter DTX 880 detection instrument was used to analyze plates run on the Biomek® FX workstation (Table 1). The GENios-Pro detection instrument is an

all-in-one solution for a broad range of primary and secondary screening applications including fluorescence intensity, flash or glow-type luminescence, and dual-color luminescence. The DTX 880 is a multimode detector that is also configured for a wide range of applications including glow-type and dual-color luminescence and fluorescence. Both are capable of reading 6- to 384-well plates and can be integrated with the corresponding liquid handling workstation.

To analyze the low-volume, high-density LV384- and 1536-well test plates, we used the BMG LABTECH PHERAstar Microplate Reader (Table 1). The PHERAstar is a multifunctional high-end reader also capable of analyzing plates with multiple readout modes including fluorescence and luminescence.

DATA and RESULTS

Multiplexed Cell Viability and Apoptosis Assays

The CellTiter-Blue® Cell Viability Assay (resazurin) was multiplexed with either the Apo-ONE® (fluorescent) or Caspase-Glo® 3/7 (luminescent) Assay. The assays were run using either suspension (Jurkat) or adherent (HEK 293) cells. Jurkat and HEK 293 cells were plated in the formats and concentrations stated in Table 2. Jurkat cells were then treated with a titration of Anti-FAS mAb from 0–500ng/ml for 5 hours at 37ºC/5% CO_2 to induce apoptosis (data not shown). This was also accomplished in HEK 293 cells by treating with varying doses of staurosporine ranging from 0–5µM for 16 hours at 37ºC/ 5% CO_2. CellTiter-Blue® Reagent was added robotically to all test plates prior to the final two hours of incubation. Fluorescence units were recorded, and either Apo-ONE® or Caspase-Glo® 3/7 Reagent was then dispensed using the various liquid handling workstations. The plates were incubated at room temperature for one hour, and fluorescence or luminescence values were recorded.

Results demonstrate successful automation of a multiplexed fluorescent viability assay with either a fluorescent or luminescent apoptosis assay in 96-, ST384-, LV384- and 1536-well formats. Figure 1 shows typical data generated from assays

Table 1. Automation Platforms and Detection Instruments Used During Multiplexed Cell-Based Assay Experiments.

Well Format	Liquid Handler	Detection Instrument
96 and ST384	Tecan Freedom EVO™	GENios-Pro
96 and ST384	Beckman Coulter Biomek® FX	DTX 880
LV384 and 1536	Deerac Fluidics™ Equator™ HTS	BMG LABTECH PHERAstar

Table 2. Cell Number Dispensed Per Well and Total Assay Volume (Per Well) for Multiplex Experiments.

Cell Line	Well Format	Cells/Well	Total Assay Volume
Jurkat	96	40,000	200µl
	384	10,000	50µl
	LV384	10,000	20µl
	1536	4,000	8µl
HEK 293	96	20,000	200µl
	384	5,000	50µl
	LV384	12,000	20µl
	1536	4,000	8µl

For assays performed in 96- and ST384-well plates, cells were plated manually. For assays performed in LV384- and 1536-well plates, cells were plated robotically.

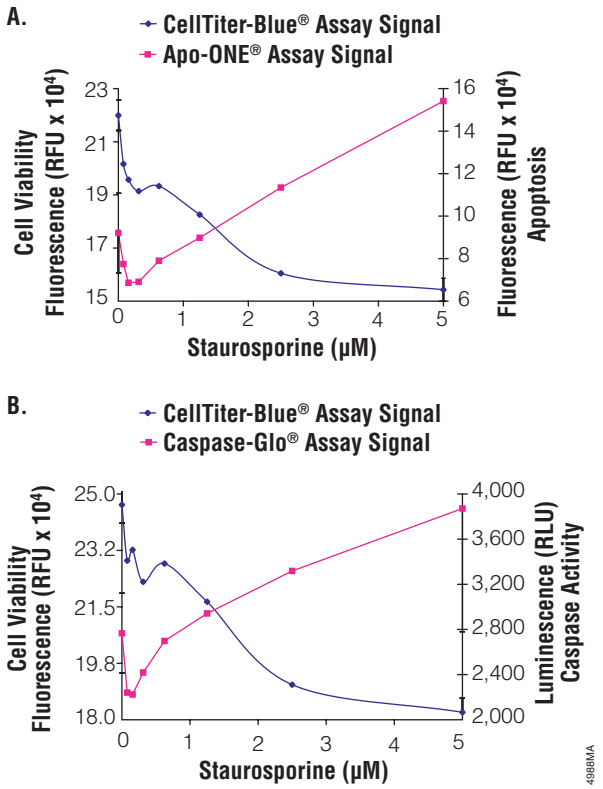

Figure 1. Coupled increase in caspase activity and decrease in cell viability measured within the same well. HEK 293 cells were plated in 1536-well plates and allowed to attach for 10 hours at 37°C/5% CO_2. Apoptosis was induced by treating with varying doses of staurosporine for 16 hours at 37°C/5% CO_2. CellTiter-Blue® Reagent was added to plates by the Equator™ HTS workstation during the final two hours of incubation. When combined with the Apo-ONE® Assay, 1X CellTiter-Blue® Reagent was added; when combined with the Caspase-Glo® 3/7 Assay the CellTiter-Blue® Reagent was diluted 1:4 in PBS. Fluorescence units were recorded, and either Apo-ONE® or Caspase-Glo® 3/7 Reagent was added by the Equator™ HTS workstation. Plates were incubated at room temperature for one hour, and fluorescence or luminescence recorded. Measurements were made using the detection instruments mentioned in Table 1.

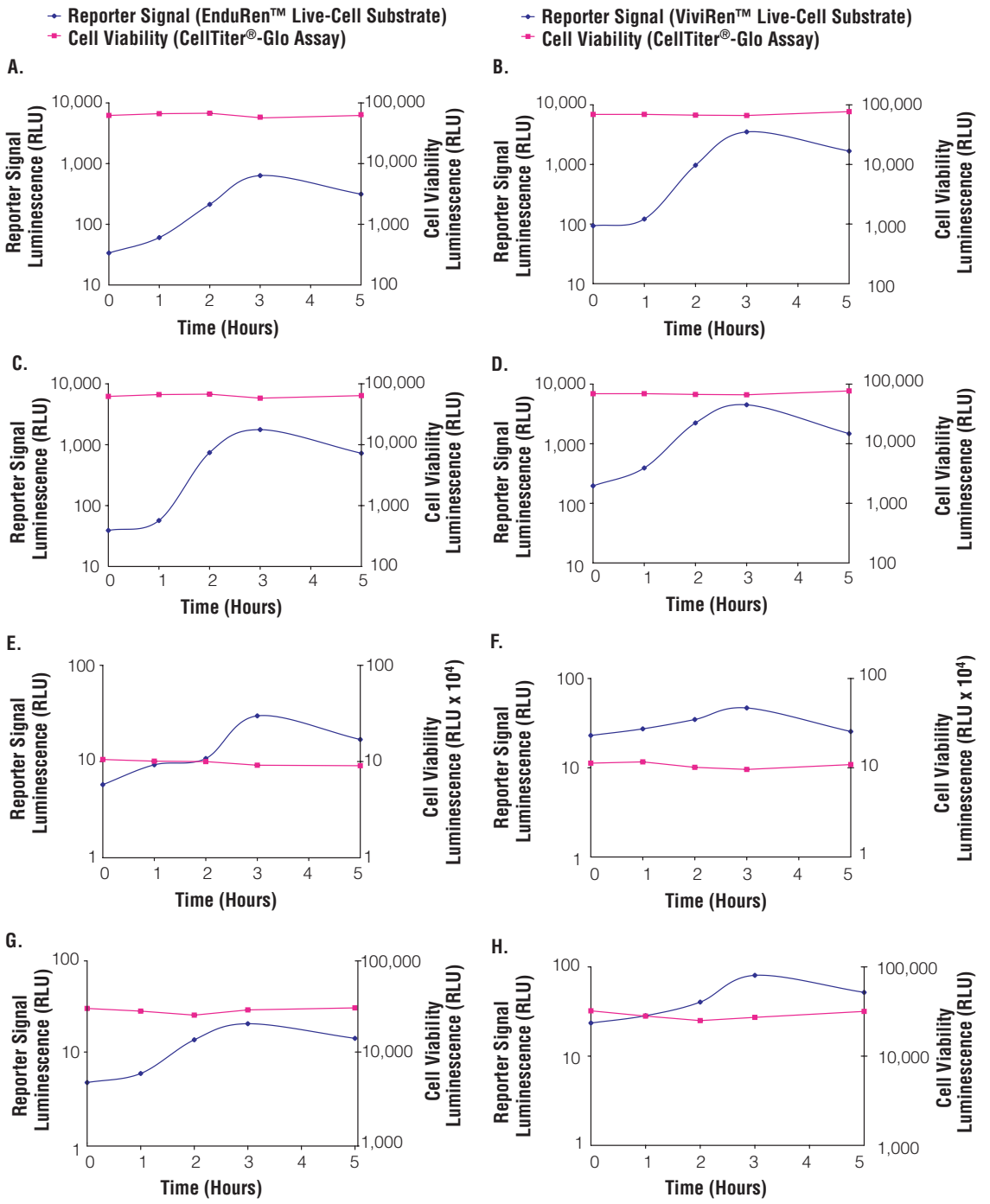

Figure 2. Measurement of CRE induction with either EnduRen™ or ViviRen™ Substrate with subsequent monitoring of cell viability within the same well. HEK 293 cells stably transfected with an inducible CRE/CL1 hPEST *Renilla* luciferase construct (2) were plated at 96-, ST384-, LV384-, and 1536-well format and allowed to attach for 10 hours at 37ºC/5% CO_2. 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. All measurements were made using the detection instruments described in Table 2. 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.** LV384-well format, Equator™ HTS workstation and PHERAstar detection instrument. **Panels E and F.** ST384-well format, Freedom EVO™ Workstation and GENios-Pro detection instrument. **Panels G and H.** 96-well format, Biomek® FX workstation and DTX 880 detection instrument.

performed in a 1536-well format. Similar data from 96-, ST384-, and LV384-well formats are available from Promega Technical Services but are not shown here.

Live Cell Reporter and Cell Viability Assay–Induction

HEK 293 cells, stably transfected with an inducible CRE/ CL1 hPEST *Renilla* luciferase construct (2) were plated in the formats and at the concentrations listed in Table 2. In test plates using EnduRen™ Live Cell Substrate (coelenterazine derivative) to measure reporter activity, the EnduRen™ Reagent was mixed with the cells and media to a final concentration of 60µM two hours prior to induction. The CRE was induced by 10µM of isoproterenol. Plates were incubated at 37ºC/5% CO_2. Reporter response and viability was assessed at the time points indicated in Figure 2. In test plates using ViviRen™ Live Cell Substrate (coelenterazine derivative), the substrate was added robotically at a final concentration of 60µM to those plates using this reagent to measure reporter activity. *Renilla* luminescence in plates containing EnduRen™ or ViviRen™ Substrate was recorded, followed by automated addition of CellTiter-Glo® Reagent to determine cell viability. Luminescence was recorded a second time to measure ATP content and cell number.

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 (Figure 2). For this application, the *Renilla* reporter expression was optimal at 3 hours of treatment with 10µM isoproterenol in all four 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.

Summary

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. The data generated in traditional 96- and ST384-well volumes, as well as in miniaturized LV384- and 1536-well formats, agrees with previous work in manual formats. The single-reagent additions, extended signal half-lives and sensitivity of each of these compatible chemistry combinations make them ideal for automated high-throughput and ultra-high-throughput liquid handling and detection. ■

This article presents only a subset of data available for automation of cell-based assays including reporter assays. For more information on automation of cell-based assays from Promega Corporation, contact Promega Technical Services Scientists at techserv@promega.com

References

1. Farfan, A. *et al.* (2004) *Cell Notes* **10**, 15–18.
2. Paguio, A. *et al.* (2005) *Promega Notes* **89**, 7–10.

Protocols

CellTiter-Glo® Luminescent Cell Viability Assay Technical Bulletin #TB288
(www.promega.com/tbs/tb288/tb288.html)

CellTiter-Blue® Cell Viability Assay Technical Bulletin #TB317
(www.promega.com/tbs/tb317/tb317.html)

Caspase-Glo® 3/7 Assay Technical Bulletin #TB323
(www.promega.com/tbs/tb323/tb323.html)

Apo-ONE® Homogeneous Caspase-3/7 Assay Technical Bulletin #TB295
(www.promega.com/tbs/tb295/tb295.html)

EnduRen™ Live Cell Substrate Technical Manual #TM244
(www.promega.com/tbs/tm244/tm244.html)

ViviRen™ Live Cell Substrate Technical Manual #TM064
(www.promega.com/tbs/tm064/tm064.html)

Ordering Information

Product	Size	Cat.#
CellTiter-Glo® Luminescent Cell Viability Assay	10 × 100ml*	G7573
CellTiter-Blue® Cell Viability Assay	10 × 100ml*	G8082
Caspase-Glo® 3/7 Assay[a]	100ml*	G8092
Apo-ONE® Homogeneous Caspase-3/7 Assay	100ml*	G7791
EnduRen™ Live Cell Substrate	34mg*	E6485
ViviRen™ Live Cell Substrate	37mg*	E6495

*Available in additional sizes.

[a]For Laboratory Use.