

Brad Larson¹, Tracy Worzella¹, and Mark Chong²

¹Promega Corporation, Madison, WI, ²Aurora Discovery, San Diego, CA

OVERVIEW

Here we demonstrate the use of Promega Corporation's luminescent, as well as fluorescent, HTS assays for profiling test compounds in a miniaturized ultra-high-throughput setting. Aurora Discovery's BioRAPTR FRD™ Workstation was used to dispense cells, test compounds, and assay reagents in a 1536-well format. IC50 calculations were performed for each compound and assay combination. Results show that IC50s from assays performed in a miniaturized uHTS setting can be used to create a clearer picture of the properties for individual compounds.

INTRODUCTION

The ability to obtain a better understanding of drug compound properties earlier in order to better predict off-target activity and toxicity is essential in the drug discovery process. By incorporating high-density plates in a miniaturized format, the researcher is able to obtain more complete information in a short period of time. High-density well formats also allow for the incorporation of a dilution series of each test compound. This enables the researcher to better understand how a compound will affect a certain target over a wide concentration range. We include cell-based assays for viability and apoptosis induction, a cell-based GPCR DRD1 assay, cytochrome P450, and kinase assays to generate each individual profile.

INSTRUMENTATION

The BioRAPTR Flying Reagent Dispenser™ (BioRAPTR FRD™) workstation was utilized in dispensing cells, compounds, ATP, enzyme/substrates, and reagents into 1536-well microplates. The BioRAPTR FRD is an automated non-contact dispenser compatible with 96, 384, 1536, and 3456-well plates. Volumes ranging from 100nL-600µL can be discretely dispensed into any well of a microplate in 10nL increments. The workstation features rapid on-the-fly dispensing, allowing all tips to address each well with simultaneous dispensing of up to 4, 8, or 16 reagents (through interchangeable head types) from individual pressurized reservoirs. The BioRAPTR FRD™ delivers reliable, high-quality performance with remarkably low dead volumes, without detectable cross contamination or carryover. An integration kit allows for flexibility with leading top-loading and articulated robot handlers and scheduling software.



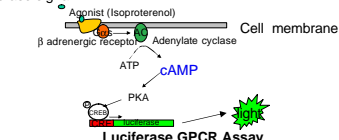
	Apo-ONE®	CytoTox-ONE™	CellTiter-Glo®	Caspase-Glo® 3/7	Dual-Glo™
Cells	2.0µl	2.0µl	2.0µl	2.0µl	1.0µl
Compd Add. 1	2.0µl	2.0µl	2.0µl	2.0µl	2.0µl
Compd Add. 2	---	---	---	---	0.5µl
Assay Reagent	4.0µl	4.0µl	4.0µl	4.0µl	3.5µl
Stop Solution	---	---	---	---	3.5µl

	P450-Glo™	Kinase-Glo® Plus
Compd Add.	2.0µl	2.0µl
Enzyme/Substr.	2.0µl	2.0µl
NADPH	1.0µl	---
ATP	---	1.0µl
Detection Reagent	5.0µl	5.0µl

Tables 1 and 2: Volumes of cells, compounds, or reagents dispensed by the BioRAPTR FRD™ for each profiling assay performed.

Methods

Dual-Glo™ Dual Luciferase GPCR Assay: The dual luciferase assay employs two plasmids. The stable cell line was established using two plasmids. One expresses firefly luciferase gene under the control of CRE/promoter and a hygromycin selectable marker and the other express a GPCR (dopamine receptor D1) and a *Renilla* luciferase-neomycin selectable marker fusion. The effects of compounds were measured by firefly luciferase signal normalized with the *Renilla* luciferase signal.

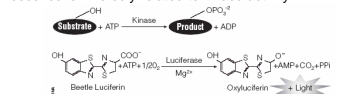


Experimental Design: Stably transfected HEK293 cells were plated in a single 1536-well plate at a concentration of 2500 cells/well. Compounds were added to all wells for the Agonist and Antagonist assays. SKF38393 was added to Antagonist assay wells 15 minutes later. The assay plate was then incubated for 4 Hrs. at 37C. Dual-Glo™ Luciferase Reagent was added, and the plate was incubated for 10 minutes before recording Luciferase RLU. Dual-Glo™ Stop & Glo® Reagent was then added, followed by similar incubation and recording of *Renilla* RLU.

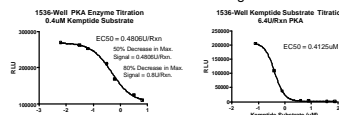
	Agonist Assay	Antagonist Assay
Nicardipine	No Induction	No Inhibition
Nifedipine	No Induction	No Inhibition
Progesterone	No Induction	No Inhibition
Desamethasone	No Induction	No Inhibition

Table 3: Compound effects on Dual-Glo™ GPCR Assays.

Kinase-Glo® Plus Assay: A universal luminescent kinase assay. The kinase reaction is conducted under the appropriate conditions. ATP remaining following the completed kinase reaction is used by the Ultra-Glo. Luciferase to catalyze the mono-oxygenation of luciferin. Luminescence is inversely related to kinase activity.



Experimental Design-Assay Optimization: Kinase and substrate were diluted across (kinase) and down (substrate) a single 1536-well plate in a 2-dimensional array. Optimal kinase was found by choosing the enzyme titration using the lowest substrate concentration that yielded >=50% change in signal over the entire titration range. The concentration which gave ~80% loss of maximum signal is the target kinase concentration. Optimal substrate was found by determining the IC50 value from the substrate titration using an excess of kinase.

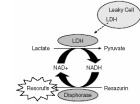


Experimental Design-Screening: Compounds (100-0uM Final Concentration), enzyme/substrate mix, and ATP (10uM) were dispensed to the plate. Kinase reactions were then incubated for 10 minutes. Detection reagent was then added, plates were incubated for an additional 10 minutes, and RLU was recorded.

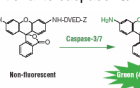
	Kinase-Glo® Plus
Nicardipine	0.075µM
Nifedipine	0.094µM
Progesterone	>100µM
Desamethasone	>100µM

Table 4: IC50 values for compounds tested with the Kinase-Glo® Plus assay.

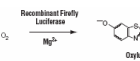
CytoTox-ONE™ Assay: A rapid, fluorescent measure of the release of lactate dehydrogenase (LDH) from cells with a damaged membrane. LDH released into the culture medium is measured with a 10-minute coupled enzymatic assay that results in the conversion of resazurin into resorufin.



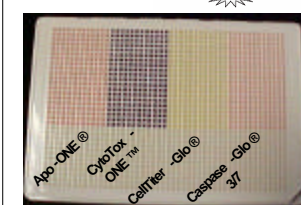
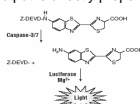
Apo-ONE®: A fluorescent, lytic assay that measures caspase-3 and -7 activity in cells undergoing apoptosis. In the presence of caspase -3 and -7, a tetrapeptide Z-DEVD-R110 substrate is cleaved, and the Rhodamine 110 leaving group becomes intensely fluorescent. Fluorescence is proportional to caspase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



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, which 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.



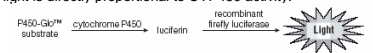
The ability to miniaturize Promega's cell-based chemistries into 1536-well format, combined with the dispensing capabilities of the BioRAPTR FRD™, enabled all four toxicity assays to be run in a single 1536-well plate.

	Apo-ONE®	CytoTox-ONE™	CellTiter-Glo®	Caspase-Glo® 3/7
4Hr. Incub.	Nicardipine >10µM: No Effect; Nifedipine >100µM: No Effect; Progesterone >100µM: No Effect; Desamethasone >100µM: No Effect	No Effect	Toxic >10µM: Non-Toxic	Toxic >10µM: 2.38µM; Non-Toxic: 0.939µM; Toxic >50µM: 0.3237µM; Non-Toxic: 0.098µM
18 Hr. Incub.	Nicardipine >10µM: No Effect; Nifedipine >10µM: No Effect; Progesterone >10µM: No Effect; Desamethasone >10µM: No Effect	No Effect	Toxic >10µM: 0.1513µM	Toxic >10µM: 0.1513µM; Non-Toxic: 0.939µM; Toxic >50µM: 0.3237µM; Non-Toxic: 0.098µM

Table 5: Toxicity values for compounds tested with cell-based viability and apoptosis assays.

Experimental Design: Jurkat cells were dispensed to a single 1536-well plate at a concentration of 2500 cells/well. Compounds were then added for either 4 or 18 hours, at concentrations ranging from 100-0uM. Assay reagents were then added, and the plate was incubated for 60 minutes. RFLU or RLU was then recorded.

P450-Glo™ Assay: A Luminescent CYP450 assay performed by incubating a luminogenic cytochrome P450 substrate with a cytochrome P450 enzyme and NADPH regeneration system. The P450-Glo™ Substrates do not react with luciferase but are converted by cytochrome P450 to luciferin, which in turn reacts with luciferase to produce light. This light is directly proportional to CYP450 activity.



Experimental Design: Compounds were dispensed to a single 1536-well plate at concentrations ranging from 100-0uM. Enzyme/substrate mix for each CYP450 isoform was then added to the plate. The plate was then incubated for 10' at room temperature. NADPH regenerating system was then added to the plate. The plate was incubated once again at RT for 60'. LDR was added, the plate was incubated for 20', and RLU was then recorded.

	1A2 Assay	2C9 Assay	3A4 Assay	2C19 Assay	2D6 Assay
CYP450 Enzyme	0.125pmol/Rxn	0.125pmol/Rxn	0.125pmol/Rxn	0.125pmol/Rxn	0.125pmol/Rxn
Substrate	100µM	100µM	50µM	25µM	25µM

	1A2 Assay	2C9 Assay	3A4 Assay	2C19 Assay	2D6 Assay
Nicardipine	14.14µM	0.05µM	1.1µM	2.3µM	6.5µM
Nifedipine	1.2µM	0.3µM	2.1µM	2.5µM	8.5µM
Progesterone	No Inhibition	0.25µM	No Inhibition	5.1µM	>100µM
Desamethasone	95.6µM	1.0µM	95.6µM	>100µM	95.6µM

Table 6: Enzyme/Substrate final assay concentrations.

Table 7: IC50 values for compounds tested with the P450-Glo™ assays. All 5 assays were run on a single 1536-well plate.

Results

	Nicardipine	Nifedipine	Progesterone	Desamethasone
Kinase-Glo® Plus	Green	Green	Green	Green
Dual-Glo™ GPCR Assay	Green	Green	Green	Green
Agonist Assay	Green	Green	Green	Green
Apo-ONE®	Green	Green	Green	Green
18 Hr. Incubation	Green	Green	Green	Green
CytoTox-ONE™	Green	Green	Green	Green
18 Hr. Incubation	Green	Green	Green	Green
CellTiter-Glo®	Green	Green	Green	Green
18 Hr. Incubation	Green	Green	Green	Green
Caspase-Glo® 3/7	Green	Green	Green	Green
18 Hr. Incubation	Green	Green	Green	Green
P450-Glo™	Green	Green	Green	Green
1A2 Assay	Green	Green	Green	Green
2C9 Assay	Green	Green	Green	Green
3A4 Assay	Green	Green	Green	Green
2C19 Assay	Green	Green	Green	Green
2D6 Assay	Green	Green	Green	Green

Table 8: Red = Inhibitory/Toxic effects <10uM. Green = Non-Inhibitory/Non-Toxic effects.

CONCLUSIONS

- Running profile screens, including both target focused and ADME-Tox assays, can provide a wealth of information about tested compounds earlier in the screening process. Each individual profile can assist in the decision process of whether or not to move forward with a compound, therefore saving both time and money.
- As large numbers of similarly structured compounds are taken through the same profile process, it becomes easier to predict the affects of each compound class.
- By miniaturizing the profiling process, it becomes easier and faster to analyze the affect of a wide range of compound concentrations, as multiple assays are able to be run in a single 1536-well plate, thereby increasing the amount of information coming from each screen.
- The combination of Promega's assays, and Aurora Discovery's BioRAPTR FRD™ non-contact dispenser, provide an ideal solution to generate the information needed for simple and efficient drug discovery.