

# High-throughput Compound Profiling Using Promega Luminescent Assays on a Tecan EVO® 200 System

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## Abstract

We demonstrate the use of Promega's luminescent HTS assays for profiling test compounds on a Tecan system. This profiling example takes a parallel approach to compound analysis by incorporating diverse assay types including cell-based assays for viability and apoptosis induction, a cell-based GPCR DRD1 assay, cytochrome P450, P-glycoprotein, monoamine oxidase, and kinase assays. Using a panel of assays, we show that one can obtain a better understanding of drug compound properties in order to better predict off-target activity and toxicity.

For this application, we have chosen several kinase (PKA) inhibitors and demonstrate the vast amount of information that can be obtained from these compounds by assaying them against a variety of chemistries. To generate the data, a Tecan Freedom Evo® 200 with an integrated TeMo™ was used to dispense cells, serially dilute test compounds and assemble assays in 384-well format. Luminescence was recorded with a Tecan GENios Pro™ plate reader. IC<sub>50</sub> or EC<sub>50</sub> calculations were performed for each compound and assay combination. Results show that data from these assays can be used to determine multiple compound characteristics for subsequent lead selection or optimization.

## General Assay Protocol

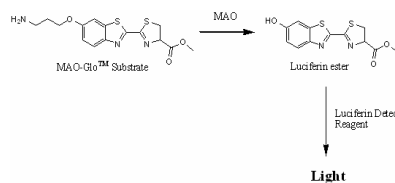
### Biochemical Assays

1. Perform serial 1:3 dilutions of test compound with 8-tip LiHa.
2. Transfer test compounds in quadruplicate to 384-well assay plates with TeMo™.
3. Dispense enzyme, substrate, and other assay components with TeMo™.
4. Move plates to shaker with RoMa arm.
5. Incubate (note: all assays were performed at 22°C, with the exception of Pgp-Glo™ and P450-Glo™ CYP2C9 assays which were performed at 37°C.)
6. Add detection reagent with TeMo™.
7. Move plates to shaker with RoMa arm.
8. Transfer plates to GENios Pro™ reader with RoMa arm.

### Cell-based Assays

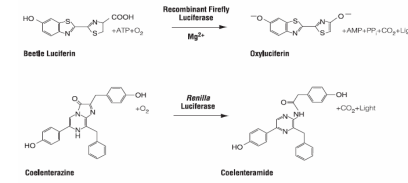
1. Dispense cells to 384-well plates with TeMo™.
2. Perform serial 1:3 dilutions of test compound with 8-tip LiHa.
3. Transfer test compounds in quadruplicate to 384-well plates with TeMo™.
4. Move plates to shaker with RoMa arm.
5. Incubate plates at 37° / 5% CO<sub>2</sub> or 37° / 10% CO<sub>2</sub>.
6. Add detection reagent to plates with TeMo™.
7. Move plates to shaker with RoMa arm.
8. Transfer plates to GENios Pro™ reader with RoMa arm.

## MAO-Glo™



**Fig. 4. The MAO-Glo™ assay for monoamine oxidase activity.** MAO-Glo™ provides a two-step luminescent assay for measuring monoamine oxidase activity. In the first step, an analog of luciferin is oxidized by MAO. In the second step, a luciferin detection reagent generates light from the product of the MAO reaction. The net amount of light generated is proportional to the activity of MAO. MAO A was used in this application, although MAO-Glo™ can be used for assessing either MAO A or MAO B activity.

## Dual-Glo™ Luciferase Assay System



**Fig. 7. The Dual-Glo™ assay sequentially measures both firefly and Renilla luciferases.** The Dual-Glo™ dual reporter system was used to assess the effect of the test compounds on the GPCR DRD1. The first step of this assay involves the measurement of the firefly luciferase and quantification of light by adding Dual-Glo™ Luciferase Reagent which generates a stable luminescent signal. Next, Dual-Glo™ Stop & Glo™ reagent is added to the same well, simultaneously quenching the firefly signal and initiating the Renilla luciferase reaction, allowing for the quantification of light from Renilla. For this application, a stable dual luciferase HEK293 cell line was used to assess compound effects on the GPCR DRD1. 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 expresses a GPCR (dopamine receptor D1) and a Renilla luciferase-neomycin selectable marker fusion.

## Summary of Cell-Based Assay Profile

Test compound	CellTiter-Glo® @ 4hrs	CellTiter-Glo® @ 18hrs	Casp-Glo® 3/7 @ 4hrs	Casp. Glo® 3/7 @ 18hrs
Dihydroxidine	IC50 > 11 µM	IC50=23.3 µM	EC50 > 11 µM	EC50 > 11 µM
H 89	IC50 = 21.3 µM	IC50 = 15.9 µM	EC50 > 3.7 µM	EC50 > 3.7 µM
PKI	No effect	No effect	No effect	No effect

**Table 3. Cell-based assay profile for viability and apoptosis induction.** Assays were performed using 5,000 Jurkat cells/well incubated in the presence of test compound for either 4 hours or 18 hours at 37°C / 5% CO<sub>2</sub>. Test compounds were serially diluted 1:3 such that the final concentration in the assay ranged from 100 µM to 0 µM for the dihydroxidine and H 89 compounds, or 1 µM to 0 µM for the PKI. IC<sub>50</sub> and EC<sub>50</sub> calculated using Graphpad Prism v4.0 software.

For the CellTiter-Glo® assay, compounds that showed toxicity at only one or two concentrations were recorded as having an IC<sub>50</sub> above the lowest concentration showing no toxicity. For the Caspase-Glo® 3/7 assay, compounds that demonstrated apoptosis induction were recorded as having an EC<sub>50</sub> above the lowest concentration showing no caspase 3/7 activity.

Results indicate that PKI has no effect on either assay tested at both 4 and 18 hour time points. Dihydroxidine and H 89 were fairly equivalent in their effects on both cell viability and apoptosis induction at both time points.

## Profile Panel

## P450-Glo™ Assay Systems

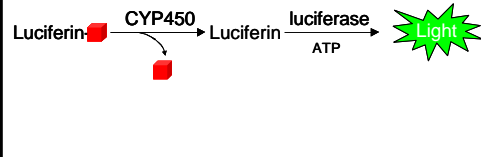
### Biochemical Assays

- P450-Glo™ CYP450 Assay Systems: CYP1A2, CYP2C9, CYP3A4, CYP2C19\* and CYP2D6\* assays
- Pgp-Glo™ Assay System
- MAO-Glo™ Assay System\* with monoamine oxidase A
- Kinase-Glo® Plus Luminescent Kinase Assay with PKA

### Cell-Based Assays

- CellTiter-Glo® Luminescent Cell Viability Assay
- Caspase-Glo® 3/7 Assay System
- Dual-Glo™ Luciferase Assay System for GPCR DRD1

The panel of 11 luminescent assays tested in this application comprise of a variety of biochemical and cell-based assay types. \* denotes products that are currently in development.



**Fig. 2. The P450-Glo™ reaction.** Modified luciferin substrates are oxidized by the cytochrome P450 enzyme, generating luciferin. The luciferase enzyme uses the luciferin substrate, ATP and oxygen to create light. In the case of the P450-Glo™ Assays, light production is directly proportional to CYP450 activity. Test compounds that inhibit CYP450 activity result in less light production.

## Kinase-Glo™ Plus



**Fig. 5. The Kinase-Glo® Plus assay for PKA.** The Kinase-Glo® assay is a simple means of measuring kinase activity by quantifying the amount of ATP left in solution following a kinase reaction. For this application, we used PKA enzyme as our target kinase, kemptide substrate and 10µM ATP in each reaction. Following a 10-minute kinase reaction with PKA, an ATP detection reagent is added and luminescence is recorded. Light signal is inversely proportional to kinase activity.

## Summary of Biochemical Assay Profile

Test compound	P450-Glo™ CYP1A2	P450-Glo™ CYP2C9	P450-Glo™ CYP3A4	P450-Glo™ CYP2C19	P450-Glo™ CYP2D6
Dihydroxidine	IC50=22.8 µM	IC50=37.7 µM	IC50 > 100 µM	IC50 > 100 µM	IC50=10.42 µM
H 89	IC50=0.53 µM	IC50 > 100 µM	IC50=0.87 µM	IC50=2.8 µM	IC50=6.1 µM
PKI	No effect	No effect	No effect	No effect	No effect

**Table 1. Cytochrome P450 profile.** Test compounds were serially diluted 1:3 such that the final concentration in the assay ranged from 100 µM to 0 µM for the dihydroxidine and H 89 compounds, or 1 µM to 0 µM for the PKI. IC<sub>50</sub> was calculated using Graphpad Prism v4.0 software.

Compounds that only showed effects at the highest concentration tested (100 µM), were recorded as having an IC<sub>50</sub> value > 100 µM.

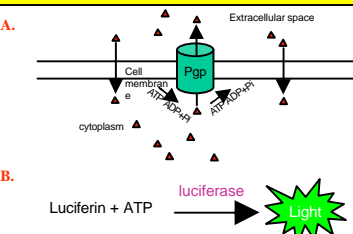
Results indicate that dihydroxidine and H 89 inhibit the various CYP450 isoenzymes with a range of intensities. Compared to a no compound control, PKI did not exhibit inhibitory or stimulatory effects on any of the CYP isoenzymes, and results were recorded as "no effect."

## Tecan Instrumentation



**Fig. 1. The Tecan Freedom EVO® 200 system with 8-tip LiHa and RoMa arm, integrated with a TeMo™ and GENios Pro™ plate reader.**

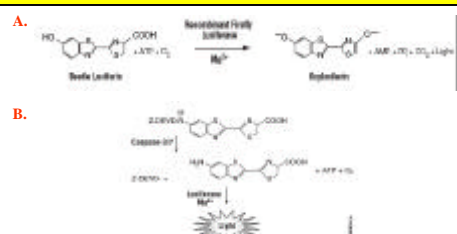
## Pgp-Glo™ Assay System



**Fig. 3. The Pgp-Glo™ luminescent ATPase assay.** Panel A: Pgp, also known as MDR1 and ABCB1, is a 170 kDa integral plasma membrane protein that functions as an ATP dependent drug efflux pump and plays an important role in multi-drug resistance and certain adverse drug-drug interactions. Compounds that are substrates for transport by Pgp stimulate its ATPase activity. By comparing basal to test compound treated samples the compounds can be ranked as stimulators or inhibitors of basal Pgp ATPase activity or as having no effect.

Panel B: The Pgp assay measures basal and drug dependent ATP consumption using the luciferase reaction. Pgp ATPase activity is inversely proportional to light output.

## CellTiter-Glo® and Caspase-Glo® 3/7



**Fig. 6. The CellTiter-Glo® and Caspase-Glo® 3/7 assays.** Panel A: The CellTiter-Glo® assay is a homogeneous (add-mix-measure) method of determining the number of viable cells present in culture by quantitating the amount of ATP present. For this application, Jurkat cells were used to examine toxic effects of the test compounds.

Panel B: The Caspase-Glo® 3/7 assay is a homogeneous method of measuring caspase activity in cells that have been induced to undergo apoptosis. A pro-luminescent Z-DEVD-luciferin substrate is cleaved in the presence of caspases 3 and 7, generating luciferin which in turn reacts with luciferase to produce light. For this application, Jurkat cells were used to examine the apoptosis inducing properties of the test compounds.

## Summary of Biochemical Assay Profile

Test compound	Kinase-Glo® PKA	MAO-Glo™ MAO A	Pgp-Glo™ P-glycoprotein
Dihydroxidine	IC50 = 2.35 µM	No effect	Stim. @ 11 µM
H 89	IC50 = 0.58 µM	IC50 = 59.24 µM	Stim. @ 33 µM
PKI	IC50 = 35.95 nM	No effect	No effect

**Table 2. Kinase, monoamine oxidase and P-glycoprotein profile.** Test compounds were serially diluted 1:3 such that the final concentration in the assay ranged from 100 µM to 0 µM for the dihydroxidine and H 89 compounds, or 1 µM to 0 µM for the PKI. IC<sub>50</sub> was calculated using Graphpad Prism v4.0 software.

For the Pgp-Glo™ assay, data is presented as the smallest test compound concentration that stimulated ATPase activity by at least 3-fold.

Results confirm that all three compounds tested are PKA inhibitors to varying degrees. Only H 89 showed an effect on monoamine oxidase A activity, while the remaining compounds showed no effect compared to the no compound control. For P-glycoprotein, dihydroxidine and H 89 were both picked up as stimulators of ATPase activity, with dihydroxidine being a slightly stronger stimulator. PKI showed no effect on Pgp ATPase activity as compared to the control.

## Summary of Cell-Based Assay Profile

Test compound	Dual-Glo™ GPCR Agonist Assay	Dual-Glo™ GPCR Antagonist Assay
Dihydroxidine	EC50 = 0.13 µM (toxic > 10 µM)	No effect
H 89	No effect (toxic > 9 µM)	No effect (toxic > 3 µM)
PKI	No effect	No effect

**Table 4. Cell-based assay profile for GPCR DRD1 effects.** Assays were performed using 5,000 HEK293 cells/well. Test compounds were serially diluted 1:3 such that the final concentration in the assay ranged from 80 µM to 0 µM for the dihydroxidine and H 89 compounds, or 0.8µM to 0 µM for the PKI. For the agonist assay, cells were incubated in the presence of test compound for 4 hours at 37°C / 10% CO<sub>2</sub>, followed by the Dual-Glo™ assay. For the antagonist assay, cells were incubated in the presence of the test compounds for 15 minutes, followed by the addition of an agonist (1µM SKF 38393 + 100µM Ro) and incubated for an additional 4 hours. The Dual-Glo™ assay was then performed. To measure compound effects, firefly RLUs were normalized to Renilla RLUs, with IC<sub>50</sub> and EC<sub>50</sub> calculated using Graphpad Prism v4.0 software.

For the agonist assay, an induction of CRE was observed with dihydroxidine only, with toxicity noted at doses above 10µM (seen as a decrease in Renilla signal at those concentrations). None of the compounds showed an antagonist response as compared to the agonist only control in that assay.

## Acknowledgements

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