**5HT1<sub>A</sub> serotonin receptor screening using β-lactamase as a reporter system**

Jasmin Gibson, Radhika Desai, Jeff Beauchaine, Randy Hoffman, and Bonnie Hanson
Invitrogen Drug Discovery Solutions • 501 Charmany Drive • Madison, WI 53719 • USA

### Introduction

Here we describe a cell-based assay platform for small molecule screening of G-protein coupled receptor (GPCR) modulators. A stable cell line expressing the 5HT1<sub>A</sub> serotonin receptor, a promiscuous G protein (Gα<sub>15</sub>), and beta-lactamase under control of the NFAT response element was created.

The assay was validated for increased beta-lactamase expression upon stimulation with known ligands as well as function in antagonist mode using a known inhibitor of the receptor. The cell line was then screened against the LOPAC™ library of small molecules, which consists of a large number of neurotransmitter like compounds. Random compounds that were detected from the screen were followed up by dose response analysis. Positive "hits" revealed a list of compounds that include known agonists for the 5HT1<sub>A</sub> serotonin receptor with a high level of receptor subtype selectivity.

Data from this screening project suggest that the beta-lactamase reporter system employed in a cell-based assay platform can produce robust (Z’-factor > 0.5), quantitative, and miniaturizable assays.

### GPCR signaling monitored by β-lactamase

Stable cell lines expressing the NFAT response element (monitoring Ca<sup>2+</sup> flux) linked to the beta-lactamase gene have been developed. These cell lines can be used as building blocks to develop specific GPCR assays. G<sub>α</sub> coupled receptors can be transfected into the Ca<sup>2+</sup>-responsive NFAT cell line. The promiscuous G protein, Gα<sub>15</sub>, can be co-transfected with G<sub>α</sub> coupled receptors into the NFAT responsive cell line to re-direct Gi coupled signaling to the G<sub>α</sub> pathway. Upon stimulation the cell lines respond with an increase in β-lactamase expression. This β-lactamase response can be quantified using a FRET-based substrate, CCF4-AM, in a fluorescence plate reader.
**Beta-lactamase assay system**

CCF4-AM is a fluorescence resonance energy transfer (FRET) based substrate for beta-lactamase. Once CCF4-AM enters a cell, it is converted to the negatively charged CCF4 by endogenous esterases. Excitation of this substrate at 409 nm leads to efficient FRET between the coumarin and fluorescein derivatives, resulting in green fluorescence detectable at 530 nm. Cleavage of CCF4 by beta-lactamase results in separation of the two fluorophores and loss of FRET, resulting in blue fluorescence detectable at 460 nm.

**Materials and Methods**

**Creation of the 5HT1A stable cell line.** CHO-K1 cells were stably transfected with the beta-lactamase (BLA) gene attached to an upstream NFAT promoter sequence using Lipofectamine™ 2000 (Invitrogen). These NFAT-BLA CHO-K1 cells were then stably transfected with the Gα15 gene followed by stable transfection of the 5HT1A receptor gene. The cells were then stimulated with 5-carboxamidotryptamine and sorted by FACS for single responsive cells. The best performing clone was chosen for the following two experiments:

**Dose responses for compounds.** For agonist assays, cells were plated in DMEM-based assay medium in 384 well plates at 20,000 cells per well. Serial dilutions of the compounds were added to the wells along with 0.5% DMSO with one set of wells having no compound added as an un-stimulated control. Plates were incubated for 5 hours in a 37°C/5% CO₂ humidified incubator. After incubation, the plates were loaded with CCF4 substrate for 2 hours at RT in the dark. Data was collected using a CytoFluor® 4000 fluorescence microplate reader at an excitation wavelength of 409 nm and two emission wavelengths of 460 and 530 nm. The results were plotted as the 460/530 ratio corresponding to each concentration of the dose response. For antagonist assays, serial dilutions of the antagonist were added, followed by the EC80 concentration of a known agonist. Results were plotted as the 460/530 ratio at each concentration of antagonist dose response.

**LOPAC1280™ library screens.** The LOPAC1280™ (LO1280) compound library consisting of 1280 compounds was screened in agonist mode using the 5HT1A cell line. Test compounds from the library were transferred from 96-well plates to 384-well black-wall, clear-bottom Costar plates (#3712). Each compound was diluted in sterile water at a concentration of 40 µM. Plates were then covered and stored at ~20°C. Assay plates prepared as described above were thawed at 4°C for a day and then for an hour at RT before the experiment. Final assay volume consisted of 10 µM test compound, 25,000 cells, and 1% DMSO in assay medium (DMEM, 2% charcoal dextran stripped FBS, 25 mM HEPES, NEAA, and sodium pyruvate). For the positive control wells 1 µM 5-CT was used. Plates were incubated in a 37°C/5% CO₂ humidified incubator for 5 hours. After 5 hours incubation, the plates were equilibrated to room temperature for 1 hour. The assay plates were then loaded with substrate (CCF4-AM) according to manufacturer’s instructions and allowed to incubate at room temperature protected from light for 2 hours. A CytoFluor® 4000 fluorescence microplate reader having an excitation wavelength of 409 nm and emission wavelengths of 460 nm and 530 nm was used for data collection.
The assay was run in agonist mode to search for compounds that trigger the Ca\(^{2+}\) flux pathway upon binding to the serotonin receptor, resulting in beta-lactamase expression. The 5HT1a cell line was able to detect serotonin ligands present in the library at 10 µM screening concentration with a high degree of subtype selectivity. From the hits ergocristine, pergolide methanesulfonate, 5-carboxamidotryptamine, and R-(+)-8-hydroxy-DPAT hydrobromide were chosen from the screen for follow-up dose response analysis. Several 5HT1a serotonin receptor subtype selective compounds were detected including: #336, N,N-Dopropyl-5-carboxamidotryptamine maleate; #443, 5-Carboxamidotryptamine maleate; #636, R-(+)-8-Hydroxy-DPAT hydrobromide; #746, Oxymetazoline hydrochloride; #1023, PAPP.

**Statistical data determined from screen**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of compounds</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hit rate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Known 5HT1a agonists detected</td>
<td>22</td>
<td>1.7</td>
</tr>
<tr>
<td>Other agonists detected</td>
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<td>1.4</td>
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<tr>
<td></td>
<td>4</td>
<td>0.3</td>
</tr>
<tr>
<td>Non-5HT1a subtype selective compounds in LOPAC(^{1280})™</td>
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<td></td>
</tr>
<tr>
<td>Non 5HT1a subtype selective compounds detected in screen</td>
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<td>1.1</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5HT1a serotonin agonist in library not detected</td>
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<td></td>
</tr>
<tr>
<td>Partial agonist</td>
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<td>0.04</td>
</tr>
<tr>
<td>Racemic compound (active isomer was detected)</td>
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<td>0.02</td>
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<tr>
<td>Other agonist</td>
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<td>0.01</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>0.01</td>
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</table>

This table contains the statistical data pertaining to the LOPAC\(^{1280}\)™ screen with 5HT1A-G\(\alpha\)15-NFAT-bla CHO-K1 stable cell line.

**Z’-factor determination of 5HT1a BLA assay from LOPAC\(^{1280}\)™ screen**

On each screening plate, 5HT1A-G\(\alpha\)15-NFAT-CHO-K1 cells were stimulated with a positive control, 1 µM 5-carboxamidotryptamine, in the presence of 0.5% DMSO for 5 hours in DMEM-based assay medium. The plates were then treated as stated in the material and method section for LOPAC screen.
**5HT1A agonist hits dose response from LOPAC™ screen**

### Dose response determination

For this assay, 5HT1A-Gα15-NFAT-bla CHO-K1 cells were stimulated with test compounds in the presence of 0.5% DMSO for 5 hours in DMEM-based assay medium. Cells were then loaded with CCF4 for 2 hours at RT. Emission data at 460 and 530 nm were collected using an excitation wavelength of 409 nm. Data were plotted as 460/530 nm ratios versus the concentration of stimulant.

### Identification of compounds and EC₅₀ values

<table>
<thead>
<tr>
<th>LOPAC™ compound no.</th>
<th>Compound name</th>
<th>Experimental EC₅₀ values (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>443</td>
<td>5-carboxamidotryptamine maleate</td>
<td>18</td>
</tr>
<tr>
<td>636</td>
<td>R-(+)-8-hydroxy-DPAT hydrobromide</td>
<td>28</td>
</tr>
<tr>
<td>467</td>
<td>Ergocristine</td>
<td>340</td>
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<tr>
<td>1047</td>
<td>Pergolide methanesulfonate</td>
<td>709</td>
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</tbody>
</table>

This table contains the EC₅₀ values determined when using the beta-lactamase assay for follow-up compound analysis from the screen.

**5HT1A-Gα15-NFAT-bla CHO-K1 antagonist dose response**

The 5HT1A assay can also be run in antagonist mode to screen for inhibitors of the 5HT1A serotonin receptor. To run this assay, 5HT1A-Gα15-NFAT-bla CHO-K1 cells were treated with the 5HT1A selective antagonist, WAY 100635, in the presence of 5-CT and 0.5% DMSO for 5 hours in DMEM based assay media. Cells were then loaded with CCF4 for 2 hours at RT. Emission data at 460 and 530 nm were collected using an excitation wavelength of 409 nm. Data were plotted as 460/530 nm ratios against the concentration of stimulant. Data obtained reveals a dose-dependent specific inhibition of the 5-CT response.
Conclusions

- We have developed a stable 5HT1A serotonin receptor cell-based assay utilizing beta-lactamase technology.
- Dose response of ligands tested in agonist and antagonist mode demonstrate accurate EC50/IC50.
- LOPAC screening data using the 5HT1A cell line demonstrate an excellent hit rate with a high degree of selectivity for 5HT1A agonists.
- Z’-factor data for the 5HT1A (0.66) cell line shows the robustness of this assay in a low volume, 384-well screening environment.