

# Mouse Peroxisome Proliferator-Activated Receptor Alpha (nr1c1, pparA, mPPARα) Reporter Assay System

**96-well Format Assays** Product # M00111

**Technical Manual** 

(version 7.2b)

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# Mouse PPARa Reporter Assay System 96-well Format Assays

I. Description
The Assay System
The Assay Chemistry3
Preparation of Test Compounds
Considerations for Automated Dispensing4
- Assay Scheme
Assay Performance5
II. Product Components & Storage Conditions
III. Materials to be Supplied by the User7
IV. Assay Protocol
A word about <i>Antagonist</i> -mode assay setup8
■ DAY 1 Assay Protocol8
■ DAY 2 Assay Protocol10
V. Related Products11
VI. Limited Use Disclosures
APPENDIX 1: Example Scheme for Serial Dilutions

#### I. Description

#### The Assay System

This nuclear receptor assay utilizes proprietary non-human cells engineered to provide constitutive, high-level expression of the Mouse Peroxisome Proliferator-Activated Receptor Alpha (nr1c1), a ligand-dependent transcription factor commonly referred to as pparA or  $mPPAR\alpha$ .

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to a PPAR $\alpha$ -responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in mPPAR $\alpha$  activity. The principal application of this assay is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against mPPAR $\alpha$ .

Mouse PPARα Reporter Cells are prepared using INDIGO's proprietary **CryoMite**<sup>TM</sup> process. This cryo-preservation method yields exceptional cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for cumbersome intermediate treatment steps such as spin-and-rinse of cells or viability determinations prior to assay setup.

INDIGO's Nuclear Receptor assays are all-inclusive cell-based assay systems. In addition to mouse PPAR $\alpha$  Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, a reference agonist, Luciferase Detection Reagent, and a cell culture-ready assay plate.

## • The Assay Chemistry •

INDIGO's nuclear receptor assay kits capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology.

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (*Photinus pyralis*). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg<sup>+2</sup>-dependent reaction that consumes O<sub>2</sub> and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP<sub>i</sub>, CO<sub>2</sub>, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer and is reported in terms of Relative Light Units (RLU's).

INDIGO's Nuclear Receptor Assay kits feature a luciferase detection reagent specially formulated to provide stable light emission between 5 and 90+ minutes after initiating the luciferase reaction. Incorporating a 5-minute reaction-rest period ensures that light emission profiles attain maximal stability, thereby allowing assay plates to be processed in batch. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.

#### Preparation of Test Compounds

Test compounds are typically solvated at high concentration in DMSO and stored frozen as master stocks. Immediately prior to setting up an assay, the master stocks are serially diluted using **Compound Screening Medium (CSM**; as described in *Step 7*) to achieve the desired assay concentrations. Do <u>not</u> use DMSO to further dilute test compound solutions. This method of dilution avoids the significant adverse effects of introducing high concentrations of DMSO into the assay. The final concentration of total DMSO carried over into assay reactions should *never* exceed 0.4%.

*NOTE:* CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup and are considered to be 'single-use' reagents.

#### Considerations for Automated Dispensing

When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument plumbing; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

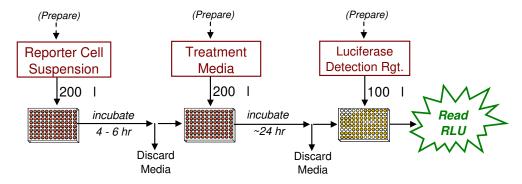
Stock Reagent & Volume provided	Volume to be Dispensed (96-well plate)	Excess rgt. volume available for instrument dead volume
Reporter Cell Suspension 21 ml (prepared from kit components)	200 μl / well 19.2 ml / plate	~ 1.8 ml
LDR 12 ml (prepared from kit components)	100 μl / well 9.6 ml / plate	~ 2.4 ml

#### ■ Assay Scheme ■

Figure 1. Assay workflow.

NOTE: This mPPAR $\alpha$  assay protocol includes Day 1 steps and dispensed volumes that differ from the historical protocol that some users may be accustomed to; please review the assay workflow, below.

In brief, 200 µl of Reporter Cells is dispensed into wells of the assay plate and pre-incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared 1x-concentration treatment media are added. Following 22-24 hr incubation, treatment media are discarded and Luciferase Detection Reagent is added. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



Page 4

#### Assay Performance

## Mouse PPARa

GW590735 Dose-Response

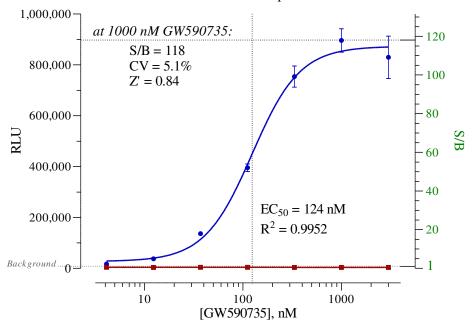


Figure 2. Dose-response of the mouse PPAR $\alpha$  Assay using reference agonist GW590735.

Dose-response analyses of mPPAR $\alpha$  Reporter Cells ( $\longrightarrow$ ) were performed according to the protocol provided in this Technical Manual. In addition, to assess the level of background signal contributed by non-specific factors that may cause activation of the luciferase reporter gene, "mock" reporter cells ( $\longrightarrow$ ) were prepared to contain only the luciferase vector (mock reporter cells are not provided with assay kits). mPPAR $\alpha$  Reporter Cells and mock reporter cells were identically treated with GW590735 (Cayman Chemical) using an assay concentration range generated in 3-fold decrements: 3000, 1000, 333, 111, 37.0, 12.3, 4.12 and 0 nM. Luminescence was quantified using a GloMax-Multi+luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration ( $n \ge 6$ ). Signal-to-background (S/B) and Z' values were calculated as described by Zhang, *et al.* (1999)<sup>1</sup>. Non-linear regression and EC<sub>50</sub> analyses were performed using GraphPad Prism software.

RESULTS: mPPAR $\alpha$  reporter cells treated with 1000 nM GW590735 yielded an S/B = 118 and a corresponding Z'= 0.84. Similarly treated mock reporter cells demonstrate no significant background luminescence ( $\leq 0.05\%$  that of the reporter cells at EC<sub>Max</sub>). Thus, luminescence results strictly through ligand-activation of the mouse PPAR $\alpha$  expressed in these reporter cells. These data confirm the robust performance of this mPPAR $\alpha$  Assay.

$$Z' = 1 - [3*(SD^{Control} + SD^{Background}) / (RLU^{Control} - RLU^{Background})]$$

<sup>&</sup>lt;sup>1</sup> Zhang JH, Chung TD, Oldenburg KR. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen.:**4** (2), 67-73.

## Mouse PPARa Assay

Alternative Agonist Dose-Response

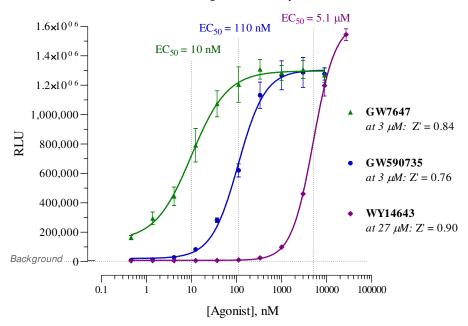


Figure 3. Dose-response analyses of mouse PPAR $\alpha$  using alternative reference agonists.

Extended dose-response analyses of mPPAR $\alpha$  Reporter Cells using alternative reference compounds GW7647 and WY14643. Concentrated stocks of all agonists were prepared in DMSO, then serially diluted in CSM.

*NOTE:* RLU values will vary slightly between different production lots of reporter cells, and can vary *significantly* between different makes and models of luminometers.

## II. Product Components & Storage Conditions

This mPPARα Assay contains materials to perform assays in a single 96-well assay plate.

The aliquot of mPPARα Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

Assay kits are shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels. Alternatively, the entire kit may be further stored at  $-80^{\circ}$ C.

To ensure maximal viability, Reporter Cells must be maintained at -80°C until immediately prior to use.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit.

Kit Components	Amount	Storage Temp.
• mPPARα Reporter Cells	1 x 2.0 mL	-80°C
- Cell Recovery Medium (CRM)	<b>2</b> x 10.5 mL	-20°C
- Compound Screening Medium (CSM)	1 x <b>45</b> mL	-20°C
• GW590735, 10 mM (in DMSO) (reference agonist for mPPARα)	1 x 30 μL	-20°C
Detection Substrate	1 x 6.0 mL	-80°C
• Detection Buffer	1 x 6.0 mL	-20°C
<ul> <li>96-well assay plate (white, sterile, cell-culture ready)</li> </ul>	1	ambient

## III. Materials to be Supplied by the User

The following materials must be provided by the user, and should be made ready prior to initiating the assay procedure:

## DAY 1

- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO<sub>2</sub> incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8- or 12-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media basins, sterile.
- sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), *or* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- Optional: antagonist reference compound.
- Optional: clear 96-well assay plate, cell culture treated, for viewing cells on Day 2.

#### **DAY 2** plate-reading luminometer.

#### IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-11* are performed on **Day 1**, requiring less than 2 hours of bench work and a 4 hr incubation step to complete. *Steps 12-17* are performed on **Day 2**, and require less than 1 hour to complete.

#### A word about Antagonist-mode assay setup

Receptor inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between  $EC_{50}-EC_{85}$ ) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This mPPAR $\alpha$  Assay kit includes a 10 mM stock solution of **GW590735**, an agonist of mPPAR $\alpha$  that may be used to setup antagonist-mode assays. 333 nM GW590735 typically approximates  $EC_{85}$  in this cell-based assay. Hence, it presents a reasonable <u>assay</u> concentration of agonist to be used when screening test compounds for inhibitory activity.

Add the challenge agonist to a bulk volume of **CSM** at an EC<sub>50</sub> – EC<sub>85</sub> concentration. This medium is then used to prepare serial dilutions of test compounds to achieve the desired respective final assay concentrations. We find that this is an efficient and precise method of setting up antagonist assays, and it is the method presented in *Step 7b* of this protocol.

**DAY 1 Assay Protocol:** All steps must be performed using aseptic technique.

- **1.)** Remove the **2 tubes** of **Cell Recovery Medium (CRM)** from freezer storage, thaw and equilibrate to  $37^{\circ}$ C using a water bath.
- **2.) Rapid Thaw of the Reporter Cells:** *First*, retrieve the two tubes of **CRM** from the 37°C water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve the tube of **Reporter Cells** from -80°C storage and, without delay, perform a rapid thaw of the frozen cells by transferring 9.5 ml from each of the 2 tubes of 37°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 21 ml

- **3.)** Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface with a 70% alcohol swab.
- **4.)** Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an 8-chanel pipette, dispense  $200~\mu l$  / well of cell suspension into the assay plate.
  - *NOTE 4.1:* Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling during the dispensing period. Likewise, take care to dispense uniform volumes across the assay plate.
  - *NOTE 4.2:* Users sometimes wish to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear 96-well cell culture treated assay plate. Continue to process the assay plate in identical manner to the white assay plate.
- **5.) Pre-incubate reporter cells:** Place the assay plate into a 37°C,  $\geq$  85% humidity, 5% CO<sub>2</sub> incubator for <u>4 6 hours</u>.

Near the end of the 4-6 hour pre-incubation period:

- **6.)** Remove **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.
- 7.) Prepare the Test Compound and Reference Compound treatment media at the desired final assay concentrations: Use CSM to prepare an appropriate dilution series of the reference and test compound stocks. Prepare all treatment media at the desired final assay concentrations. In *Step 9*, the prepared treatment media will be dispensed at 200  $\mu$ l / well into the assay plate. Manage dilution volumes carefully; this assay kit provides 45 ml of CSM.

NOTE: Total DMSO carried over into assay reactions should never exceed 0.4%.

*a. Agonist*-mode assays. This PPARα Assay kit includes a 10 mM stock solution of the reference agonist **GW590735**. The following 7-point treatment series, with concentrations presented in 3-fold decrements, provides a suitable dose-response: 3000, 1000, 333, 111, 37.0, 12...3, and 4.12 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

~ or ~

- **b.** Antagonist-mode assays. When setting antagonist assays, first supplement a bulk volume of CSM with the challenge agonist GW590735 to achieve the desired final assay-concentration (refer to "A word about antagonist-mode assay setup", pg. 8). The agonist-supplemented CSM is then used to generate dilutions of test compound samples to achieve their final assay concentrations.
- **8.**) At the end of the cell pre-incubation period, **discard the culture media** by ejecting it into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.
- 9.) Dispense 200 µl of each treatment media into appropriate wells of the assay plate.
- **10.**) Transfer the assay plate into a 37°C, humidified 5% CO<sub>2</sub> incubator for <u>22 24 hours</u>.

  NOTE: Ensure a high-humidity (≥ 85%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.
- 11.) For greater convenience on Day 2, retrieve **Detection Substrate** *and* **Detection Buffer** from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.

- **DAY 2 Assay Protocol:** Subsequent manipulations do *not* require special regard for aseptic technique, and may be performed on an open bench top.
- **12.**) 30 minutes before intending to quantify receptor activity, remove **Detection Substrate** and **Detection Buffer** from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature. Once at room temperature, gently invert each tube several times to ensure homogenous solutions.
  - *NOTE:* Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.
- 13.) Set the plate-reader to "luminescence" mode. Set the instrument to perform a single  $\underline{5}$  second "plate shake" prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, *or less*.
- **14.**) *Immediately before proceeding to Step 15*, transfer the entire volume of Detection Buffer into the vial of Detection Substrate, thereby generating a <u>12 ml</u> volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.
- **15.**) Following 22 24 hours incubation in treatment media, discard the media contents by ejecting it into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.
- **16.)** Add  $\underline{100 \, \mu l}$  of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for at least  $\underline{5 \, \text{minutes}}$  following the addition of LDR. Do not shake the assay plate during this period.
- 17.) Quantify luminescence.

## V. Related Products

Human PPARα Assay Products		
Product No.	Product Descriptions	
IB00111-32	Human PPARα Reporter Assay System 3x 32 assays in 96-well format	
IB00111	Human PPARα Reporter Assay System 1x 96-well format assay	
IB00112	Human PPARα Reporter Assay System 1x 384-well format assays	
Bulk volumes of Assay Reagents may be custom manufactured to accommodate any		

scale of HTS. Please Inquire.

Panel of Human PPAR Assays	
Product No.	Product Description
IB00131-32P	Human PPARγ, PPARα and PPARδ Reporter Assay PANEL 32 assays each in 1x 96-well plate

MOUSE PPARα Assay Products	
Product No.	Product Descriptions
M00111-32	Mouse PPARα Reporter Assay System 3x 32 assays in 96-well format
M00111	Mouse PPARα Reporter Assay System 1x 96-well format assay

RAT PPARα Assay Products	
Product No.	Product Descriptions
R00111-32	Rat PPARα Reporter Assay System 3x 32 assays in 96-well format
R00111	Rat PPARα Reporter Assay System 1x 96-well format assay

LIVE Cell Multiplex (LCM) Assay	
Product No.	Product Descriptions
LCM-01	Reagent volumes sufficient to perform <b>96</b> Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in 5x-bulk volume to perform <b>480</b> Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats
LCM-10	Reagent in 10x-bulk volume to perform <b>960</b> Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats

Please refer to INDIGO Biosciences website for updated product offerings.

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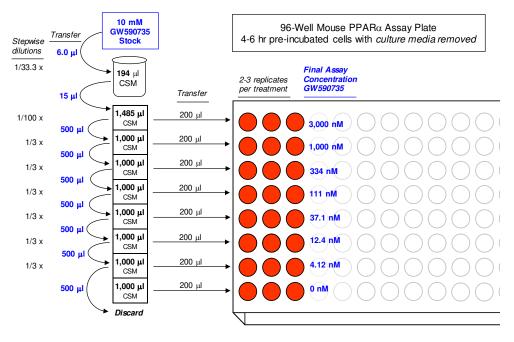
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## **APPENDIX 1**

Example scheme for the serial dilution of GW590735 reference agonist, and the setup of a mPPAR $\alpha$  dose-response assay.



Page 12