Human Peroxisome Proliferator-Activated Receptor Alpha
(NR1C1, PPARA, PPARα)
Reporter Assay System

3x 32 Assays in 96-well Format
Product # IB00111-32

Technical Manual
(version 7.2)

www.indigobiosciences.com
3006 Research Drive, Suite A1, State College, PA, 16801, USA

Customer Service:
814-234-1919; FAX 814-272-0152;
customerserv@indigobiosciences.com

Technical Service:
814-234-1919
techserv@indigobiosciences.com
Human PPARα Reporter Assay System
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I. Description

• The Assay System •

This nuclear receptor assay utilizes proprietary non-human cells engineered to provide constitutive, high-level expression of the Human Peroxisome Proliferator-Activated Receptor Alpha (NR1C1), a ligand-dependent transcription factor commonly referred to as PPARα or PPARα.

INDIGO’s Reporter Cells include the luciferase reporter gene functionally linked to a PPARα-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in PPARα 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 human PPARα.

PPARα Reporter Cells are prepared using INDIGO’s proprietary CryoMite™ 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 PPARα 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 Mg²⁺-dependent reaction that consumes O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PPi, CO₂, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer, and is reported in terms of Relative Light Units (RLU’s).

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 not 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.

• Assay Scheme •

Figure 1. Assay workflow.

NOTE: PPARα assay protocols includes Day 1 steps and dispensed volumes that are different from the conventional INDIGO assay protocol that some users may be accustomed to when setting up INDIGO's other Nuclear Receptor Assays.

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.
**Assay Performance**

Figure 2. Agonist dose-response analyses of Human PPAR α.

Analyses of PPAR α Reporter Cells using GW590735 (provided), GW7647 and WY14643 (Tocris). 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, which contain only the luciferase vector, were treated with GW590735 (mock reporter cells are not provided with assay kits). Final assay concentrations for each agonist were: 40000, 10000, 2500, 625, 156, 39.1, 9.77, 2.44, 0.600, 0.160, 0.0400, 0.01000, and 0 nM. (40,000 nM treatments of GW7647 and GW590735 induced a cytotoxic response, and these data points were omitted.) The highest assay concentration of DMSO was 0.1%. 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 ≥ 6). Signal-to-background (S/B) and Z’ values were calculated as described by Zhang, et al. (1999)\(^1\). Non-linear regression and EC\(_{50}\) analyses were performed using GraphPad Prism software. Mock reporter cells demonstrate no significant background luminescence (≤ 0.1% that of the reporter cells at EC\(_{Max}\)). Thus, luminescence results strictly through ligand-activation of PPAR α expressed in these reporter cells. Z’ scores confirm the robust performance of this PPAR α Assay.


\[Z' = 1 - \frac{3 \times (SD_{Control} + SD_{Background})}{(RLU_{Control} - RLU_{Background})}\]
II. Product Components & Storage Conditions

This Human PPARα Assay kit contains materials to perform three distinct groups of assays in a 96-well plate format. Reagents are configured so that each group will comprise 32 assays. If desired, however, individual aliquots of reagents may be combined to perform either 64 or 96 assays.

Each aliquot of 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. For simplicity, however, the entire kit may be further stored at -80°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.

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα Reporter Cells</td>
<td>3 x 0.6 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Cell Recovery Medium (CRM)</td>
<td>2 x 10.5 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Compound Screening Medium (CSM)</td>
<td>1 x 45 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>GW590735, 10 mM (in DMSO) (reference agonist for PPARα)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Detection Substrate</td>
<td>3 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Detection Buffer</td>
<td>3 x 2.0 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Plate frame</td>
<td>1</td>
<td>ambient</td>
</tr>
<tr>
<td>Snap-in 8-well strips (white, sterile, cell culture treated wells)</td>
<td>12</td>
<td>ambient</td>
</tr>
</tbody>
</table>

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% CO₂ 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 PPAR$\alpha$ Assay kit includes a 10 mM stock solution of GW590735, an agonist of PPAR$\alpha$ that may be used to setup antagonist-mode assays. 10 nM GW590735 typically approximates EC$_{50}$ in this cell-based reporter assay. Hence, it presents a reasonable assay 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$_{50}$ – EC$_{85}$ 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 PPAR$\alpha$ 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°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 **Reporter Cells** from -80°C storage: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. *Without delay*, perform a rapid thaw of the frozen cells by transferring **6.4 ml** of pre-warmed CRM into each tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **7.0 ml** per tube.

3.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface with a 70% alcohol swab.

4.) If more than one tube of Reporter Cells is thawed, combine them and gently invert several times to disperse cell aggregates and gain a homogenous cell suspension. Dispense **200 µl / 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$_2$ incubator for 4 - 6 hours.
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 µl / 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: 300, 100, 33.3, 11.1, 3.70, 1.23, and 0.412 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. 7). 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.

   Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using an 8-pin manifold (e.g., Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus. Do not touch the well bottom, or run the tip of the aspiration device around the bottom circumference of the assay well. Such practices will result in destruction of the reporter cells and greatly increased well-to-well variability.

9.) Dispense 200 µl of each prepared control media and treatment media into appropriate wells of the assay plate.

10.) Transfer the assay plate into a 37°C, humidified 5% CO2 incubator for 22 - 24 hours.

   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 the appropriate number of vials of 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 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: To read 32 assay wells, transfer the entire volume of 1 vial of Detection Buffer into 1 vial of Detection Substrate, thereby generating a 4 ml volume of Luciferase Detection Reagent (LDR). Mix gently to avoid foaming.

15.) Following 22 - 24 hours incubation in treatment media, remove media contents from each well of the assay plate (as before in Step 8).

16.) Add 100 µl of LDR to each well of the assay plate. Allow the assay plate to rest at room temperature for at least 5 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

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00111-32</td>
<td>3x 32 Human PPARα assays; strip-wells in 96-well plate frame</td>
</tr>
<tr>
<td>IB00111</td>
<td>1x 96-well format Human PPARα assays</td>
</tr>
<tr>
<td>IB00112</td>
<td>1x 384-well format Human PPARα assays</td>
</tr>
</tbody>
</table>

Bulk volumes of Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

#### Panel of Human PPAR Assays

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>IB00131-32P</td>
<td>Human PPARγ, PPARα and PPARδ Reporter Assay PANEL 32 assays each in 1x 96-well plate</td>
</tr>
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#### MOUSE PPARα Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
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<tbody>
<tr>
<td>M00111-32</td>
<td>3x 32 Mouse PPARα assays; strip-wells in 96-well plate frame</td>
</tr>
<tr>
<td>M00111</td>
<td>1x 96-well format Mouse PPARα assays</td>
</tr>
</tbody>
</table>

#### RAT PPARα Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
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<tbody>
<tr>
<td>R00111-32</td>
<td>3x 32 Rat PPARα assays; strip-wells in 96-well plate frame</td>
</tr>
<tr>
<td>R00111</td>
<td>1x 96-well format Rat PPARα assays</td>
</tr>
</tbody>
</table>

#### LIVE Cell Multiplex (LCM) Assay

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>LCM-01</td>
<td>Reagent volumes sufficient to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-05</td>
<td>Reagent in 5x-bulk volume to perform 480 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagent in 10x-bulk volume to perform 960 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
</tbody>
</table>

Please refer to INDIGO Biosciences website for updated product offerings.

[www.indigobiosciences.com](http://www.indigobiosciences.com)
VI. Limited Use Disclosures

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans.

“CryoMite” is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA)

Product prices, availability, specifications and claims are subject to change without prior notice.

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APPENDIX 1

Example scheme for the serial dilution of GW590735 reference agonist, and the setup of a PPARα dose-response assay.
Human Peroxisome Proliferator-Activated Receptor Alpha
(NR1C1, PPARA, PPARα)
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96-well Format Assays
Product # IB00111

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• 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 not 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.

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

• Assay Scheme •

Figure 1. Assay workflow.

NOTE: This PPARα 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, which is then 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.
Figure 2. Agonist dose-response analyses of Human PPARα.
Analyses of PPARα Reporter Cells using GW590735 (provided), GW7647 and WY14643 (Tocris). 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, which contain only the luciferase vector, were treated with GW590735 (mock reporter cells are not provided with assay kits). Final assay concentrations for each agonist were: 40000, 10000, 2500, 625, 156, 39.1, 9.77, 2.44, 0.600, 0.160, 0.0400, 0.01000, and 0 nM. (40,000 nM treatments of GW7647 and GW590735 induced a cytotoxic response, and these data points were omitted.) The highest assay concentration of DMSO was 0.1%. 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 ≥ 6). Signal-to-background (S/B) and Z’ values were calculated as described by Zhang, et al. (1999)1. Non-linear regression and EC50 analyses were performed using GraphPad Prism software. Mock reporter cells demonstrate no significant background luminescence (≤ 0.1% that of the reporter cells at ECMax). Thus, luminescence results strictly through ligand-activation of PPARα expressed in these reporter cells. Z’ scores confirm the robust performance of this PPARα Assay.


\[
Z' = 1 - \frac{3 \times (SD_{Control} + SD_{Background})}{(RLU_{Control} - RLU_{Background})}
\]
II. Product Components & Storage Conditions

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

The aliquot of PPARα 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°C.

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

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<td>GW590735, 10 mM (in DMSO) (reference agonist for PPARα)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Detection Substrate</td>
<td>1 x 6.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Detection Buffer</td>
<td>1 x 6.0 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>96-well assay plate</td>
<td>1</td>
<td>ambient</td>
</tr>
</tbody>
</table>

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

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- 37°C water bath.
- 70% alcohol wipes
- 8-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 a 4-6 hr pre-incubation step. *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 EC50 – EC85) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This PPARα Assay kit includes a 10 mM stock solution of **GW590735**, an agonist of PPARα that may be used to setup antagonist-mode assays. 10 nM GW590735 typically approximates EC50 in this cell-based reporter assay. Hence, it presents a reasonable assay 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 EC50 – EC85 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°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-channel pipette, dispense **200 µl / 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, ≥ 85% humidity, 5% CO₂ incubator for **4 - 6 hours**.
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 µl / 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: 300, 100, 33.3, 11.1, 3.70, 1.23, and 0.412 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. 7). 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% CO₂ incubator for 22 - 24 hours.

    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 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 12 ml 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 100 µl of LDR to each well of the assay plate. Allow the assay plate to rest at room temperature for at least 5 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

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00111-32</td>
<td>3x 32 Human PPARα assays; strip-wells in 96-well plate frame</td>
</tr>
<tr>
<td>IB00111</td>
<td>1x 96-well format Human PPARα assays</td>
</tr>
<tr>
<td>IB00112</td>
<td>1x 384-well format Human PPARα assays</td>
</tr>
</tbody>
</table>

Bulk volumes of Assay Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

### Panel of Human PPAR Assays

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00131-32P</td>
<td>Human PPARγ, PPARα and PPARδ Reporter Assay PANEL 32 assays each in 1x 96-well plate</td>
</tr>
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</table>

### MOUSE PPARα Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>M00111-32</td>
<td>3x 32 Mouse PPARα assays; strip-wells in 96-well plate frame</td>
</tr>
<tr>
<td>M00111</td>
<td>1x 96-well format Mouse PPARα assays</td>
</tr>
</tbody>
</table>

### RAT PPARα Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>R00111-32</td>
<td>3x 32 Rat PPARα assays; strip-wells in 96-well plate frame</td>
</tr>
<tr>
<td>R00111</td>
<td>1x 96-well format Rat PPARα assays</td>
</tr>
</tbody>
</table>

### LIVE Cell Multiplex (LCM) Assay

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCM-01</td>
<td>Reagent volumes sufficient to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-05</td>
<td>Reagent in 5x-bulk volume to perform 480 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagent in 10x-bulk volume to perform 960 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
</tbody>
</table>

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com


**VI. Limited Use Disclosures**

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans.

“CryoMite” is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA)

Product prices, availability, specifications and claims are subject to change without prior notice.

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**APPENDIX 1**

Example scheme for the serial dilution of GW590735 reference agonist, and the setup of a PPARα dose-response assay.
Human Peroxisome Proliferator-Activated Receptor Alpha
(NR1C1, PPARA, PPARα)
Reporter Assay System

384-well Format Assays
Product # IB00112

Technical Manual
(version 8.0b)

www.indigobiosciences.com

3006 Research Drive, Suite A1, State College, PA 16801, USA

Customer Service:
814-234-1919; FAX 814-272-0152
customerserv@indigobiosciences.com

Technical Service:
814-234-1919
techserv@indigobiosciences.com
Human PPARα Reporter Assay System
384-well Format Assays

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APPENDIX 1b: Example Scheme for Serial Dilutions when using acoustic dispensing of test compounds .................................... 12
**I. Description**

- **The Assay System**

This nuclear receptor assay system utilizes proprietary non-human cells engineered to provide constitutive, high-level expression of the **Human Peroxisome Proliferator-Activated Receptor Alpha (NR1C1)**, a ligand-dependent transcription factor commonly referred to as PPARα or PPARα.

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

PPARα Reporter Cells are prepared using INDIGO’s proprietary **CryoMite™** 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, viability determinations, cell titer adjustments, or pre-incubation of reporter cells prior to assay setup.

INDIGO's Assay kits provide the convenience of an all-inclusive cell-based assay system. In addition to PPARα Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples. Also included is the reference agonist GW590735, Luciferase Detection Reagents, and a cell culture-ready assay plate.

- **The Assay Chemistry**

INDIGO’s nuclear receptor reporter assays 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^{2+}-dependent reaction that consumes O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_{i}, CO₂, 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 Assays feature a luciferase detection reagent specially formulated to provide stable light emission between 30 and 100+ minutes after initiating the luciferase reaction. Incorporating a 30 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.
• Considerations for the Preparation and Automated Dispensing of Test compounds •
Small molecule compounds are typically solvated at high concentration (ideally 1,000x-concentrated) in DMSO and stored frozen as master stocks. For 384-well format assays these master stocks will be diluted by one of two alternative methods, the selection of which will be dictated by the type of dispensing instrument that is to be used. This Technical Manual provides detailed protocols for each of these two alternative methods:

a.) Assay setups in which a conventional tip-based instrument is used to dispense test compounds into assay wells (in black text). Use Compound Screening Medium (CSM) to generate a series of 2x-concentration test compound treatment media, as described in Step 2a of the Assay Protocol. The final concentration of DMSO carried over into assay reactions should never exceed 0.4%; strive to use 1,000x-concentrated stocks when they are prepared in DMSO.

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.

and,

b.) Assay setups in which an acoustic transfer device is used to dispense test compounds into assay wells (text highlighted in blue). Use DMSO to make a series of 1,000x-concentrated test compound stocks that correspond to each desired final assay concentrations, as described in Step 2b of the Assay Protocol.

• Considerations for Automated Dispensing of Other Assay Reagents •
When dispensing into a small number of assay plates, first carefully consider the dead volume requirement of your tip-based dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will not be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses on a per kit basis. Always pool the individual reporter cell suspensions and all other respective assay kit reagents before processing multiple 384-well assay plates.

<table>
<thead>
<tr>
<th>Stock Reagent &amp; Volume provided</th>
<th>Volume to be Dispensed (384-well plate)</th>
<th>Excess rgt. volume available for instrument dead volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>when using tip dispensing of test cmpds Reporter Cell Suspension 7.5 ml</td>
<td>15 µl / well 5.8 ml / plate</td>
<td>~ 1.7 ml</td>
</tr>
<tr>
<td>when using acoustic dispensing of test cmpds Reporter Cell Suspension 15 ml</td>
<td>30 µl / well 11.5 ml / plate</td>
<td>~ 3.4 ml</td>
</tr>
<tr>
<td>Detection Substrate 7.8 ml</td>
<td>15 µl / well 5.8 ml / plate</td>
<td>~ 2 ml</td>
</tr>
</tbody>
</table>
The Day 1 preparation, volumes, and chronology of dispensed cells and test compounds are different between assay setups using a *tip-based dispenser* (1a) and those using an *acoustic transfer device* (1b). Following 22-24 hr incubation Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.

**Figure 1a.** Assay workflow if using conventional *tip-based* dispensing of test compounds.

**Figure 1b.** Assay workflow if using *acoustic* dispensing of test compounds.

**Assay Performance**

**Human PPARα Agonist Assay**

![Graph showing agonist dose-response of PPARα.](Image)

GW590735

$EC_{50} \approx 18 \text{nM}$

Hill Slope = 1.3

$Z' = 0.70$

$\text{Fold-Activation}$ vs. $[\text{GW590735}], \text{nM}$

Figure 2. **Agonist dose-response of PPARα.** Human PPARα Reporter Cells were treated with the reference agonist GW590735 (provided). Average Relative Light Units (RLU) and their respective values of Standard Deviation (SD), Coefficient of Variation (CV), and Fold-Activation (i.e., S/B) were calculated for each treatment concentration (n = 4). $Z'$ was calculated as per Zhang, et al. (1999)$^1$.

The treatment concentrations of GW590735 were Log10 transformed and respective RLU values were normalized as Fold-Activation. Data were plotted via non-linear regression and $EC_{50}$ determined using GraphPad Prism software.


$Z' = 1 - \left[3\times (SD_{\text{Reference}} + SD_{\text{Vehicle Bkg}}) / (RLU_{\text{Reference}} - RLU_{\text{Vehicle Bkg}})\right]$
II. Product Components & Storage Conditions

This assay kit contains materials to perform assays in a single 384-well assay plate.

**Cryopreserved mammalian cells are temperature sensitive! To ensure maximal viability the tube of Reporter Cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in this protocol.**

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first inventory the individual kit components be sure to first transfer and submerge the tube of cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, the cells can NOT be refrozen. Nor can they be maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

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

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPARα Reporter Cells</td>
<td>1 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Cell Recovery Medium (CRM)</td>
<td>1 x 7 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Compound Screening Medium (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>GW590735, 300 µM (in DMSO) (reference agonist for PPARα)</td>
<td>1 x 80 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Detection Substrate</td>
<td>1 x 7.8 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>384-well assay plate (white, sterile, cell-culture ready)</td>
<td>1</td>
<td>ambient</td>
</tr>
</tbody>
</table>

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**
- dry ice container
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8-channel electronic, repeat-dispensing pipettes & tips suitable for dispensing 15 µl.
- disposable media basins, sterile.
- sterile multi-channel media basins or deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- antagonist reference compound (optional).

**DAY 2** plate-reading luminometer.
IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. Steps 1-8 are performed on Day 1, requiring less than 2 hours to complete. Steps 9-13 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 varying concentrations of the test compound(s) to be evaluated for antagonist activity. This assay kit includes a 300 µM stock solution of GW590735, an agonist of PPARα (Fig. 2) that may be used to setup antagonist-mode assays. 50 nM GW590735 typically approximates EC$_{70}$–$80$ in this assay. Hence, it presents a reasonable final assay concentration of agonist to be used when screening test compounds for inhibitory activity.

Adding the reference agonist to the bulk suspension of Reporter Cells (i.e., prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in Step 5b of the protocol when performing tip-based dispensing, and Step 6b of the protocol when using an acoustic transfer device to dispense test compounds.

Note that when using a tip-based instrument for the dispensing of 2x-concentrated test compounds the cell suspension must also be supplemented with a 2x-concentration of the challenge agonist.

When using an acoustic transfer device for the dispensing of 1,000x-concentrated test compounds the cell suspension should be supplemented with a 1x-concentration of the challenge agonist.

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

1.) Remove Cell Recovery Medium (CRM) and Compound Screening Medium (CSM) from freezer storage and thaw in a 37°C water bath.

2.) Prepare dilutions of treatment compounds: Prepare Test Compound treatment media for Agonist- or Antagonist-mode screens. NOTE that test and reference compounds will be prepared differently when using tip-dispensing vs. acoustic dispensing. Regardless of the method, the total DMSO carried over into assay reactions should never exceed 0.4%.

a. Tip dispensing method: In Step 6, 15 µl / well of the prepared treatment media is added to the assay that has been pre-dispensed with 15 µl /well of Reporter Cells. Hence, to achieve the desired final assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Use CSM to prepare the appropriate dilution series. Plan dilution volumes carefully; this assay kit provides 35 ml of CSM.

b. Acoustic dispensing method: In Step 6, 30 nl / well of 1,000x-concentrated test compound solutions (prepared in DMSO) are added to the assay plate using an acoustic transfer device.

Preparing the positive control: This assay kit includes a 300 µM stock solution of GW590735, a common reference agonist of PPARα. The following 7-point treatment series, with concentrations presented in 3-fold decrements, provides a complete dose-response: 300, 100, 33.3, 11.1, 3.70, 1.23, and 0.412 nM. Always include a 'no treatment' (or 'vehicle') control.

APPENDIX 1a provides an example for generating such a dilution series to be used when tip-dispensing compound solutions prepared in CSM (15 µl / well).

APPENDIX 1b provides an example for generating such a series of 1,000x-concentrated solutions of compounds prepared in DMSO to be used when performing acoustic dispensing (30 nl / well).
When using tip-based instrumentation for dispensing test compounds …

3.) First, retrieve the tube of CRM from the 37°C water bath, sanitize the outside with a 70% ethanol swab;

Second, retrieve Reporter Cells from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. Perform a rapid thaw of the frozen cells by transferring a 5.5 ml volume of 37°C CRM into the tube of frozen cells. Recap the tube of Reporter Cells and place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.5 ml.

4.) Retrieve the tube of Reporter Cell Suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab, then transfer it into the cell culture hood.

5.) Gently invert the tube of cell suspension several times to disperse cell aggregates and gain a homogenous suspension.

a. for Agonist-mode assays: Dispense 15 µl / well of cell suspension into the Assay Plate.

b. for Antagonist-mode assays: Supplement the bulk volume of Reporter Cells suspension with a 2x-concentration of the challenge agonist (refer to "A word about antagonist-mode assay setup", pg. 7). Dispense 15 µl / well of cell suspension into the Assay Plate.

6.) Dispense 15 µl / well of 2x-concentrated treatment media (from Step 2a) into the assay plate.

When using an acoustic transfer device for dispensing test compounds …

3.) Dispense 30 nl / well of the 1,000x-concentrated compounds (in DMSO solutions, from Step 2b) into the assay plate.

4.) First, retrieve the tube of CRM from the 37°C water bath, sanitize the outside with a 70% ethanol swab;

Second, retrieve Reporter Cells from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. Perform a rapid thaw of the frozen cells by transferring a 5.5 ml volume of 37°C CRM into the tube of frozen cells. Recap the tube of cells and place it in a 37°C water bath for 5 - 10 minutes.

5.) Retrieve the tube of cell suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab. Add an additional 7.5 ml of CSM to the tube. The resulting volume of cell suspension will be 15 ml.

6.) Gently invert the tube of cells several times to disperse cell aggregates and gain a homogenous cell suspension.

a. for Agonist-mode assays: Dispense 30 µl / well of cell suspension into the Assay Plate that has been pre-dispensed with test compounds.

b. for Antagonist-mode assays: First supplement the bulk volume of PPARα Reporter Cells suspension with the challenge agonist GW5907375 to achieve an EC<sub>50</sub> – EC<sub>80</sub> concentration (refer to "A word about antagonist-mode assay setup", pg. 7). Then dispense 30 µl / well of the supplemented cell suspension into the assay plate that has been pre-dispensed with test compounds.

NOTE: Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate will cause well-to-well variation (= increased Standard Deviation) in the assay.

(continued …)
NOTE: Following the dispensing of Reporter Cells and test compounds INDIGO recommends performing a low-speed spin of the assay plate (with lid) for 1-2 minutes using a room temperature centrifuge fitted with counter-balanced plate carriers.

7.) Transfer the assay plate into a 37°C, humidified, 5% CO₂ incubator for 22 - 24 hours.
   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.

8.) For greater convenience on Day 2, retrieve Detection Substrate from freezer storage and place 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 a bench top.

9.) Approximately 30 minutes before intending to quantify receptor activity remove Detection Substrate from the refrigerator and place it in a low-light area so that it may equilibrate to room temperature. Gently invert the tube several times to ensure a homogenous solution.
   NOTE: Do NOT actively warm Detection Substrate above room temperature. If this solution was not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.

10.) Set the plate-reader to “luminescence” mode. Set the instrument to perform a single 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.

11.) Following 22 - 24 hours of incubation dispense 15 µl / well of Detection Substrate to the assay plate.
   NOTE: Perform this reagent transfer carefully to avoid bubble formation! Scattered micro-bubbles will not pose a problem. However, bubbles covering the surface of the reaction mix, or large bubbles clinging to the side walls of the well, will cause lens-effects that will degrade the accuracy and precision of the assay data. INDIGO recommends performing a final low-speed spin of the assay plate (with lid) for 1-2 minutes using a room temperature centrifuge fitted with counter-balanced plate carriers.

12.) Allow the plate(s) to rest at room temperature for 30 minutes. Do not shake the assay plate(s) during this period.
   NOTE: the luminescent signal is unstable during the first 30 minutes of the luciferase reaction, however, after the initial 30 minute reaction period the luminescence signal achieves a stable emission output.

13.) Quantify luminescence.
### V. Related Products

#### Human PPARα Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
</table>
| IB00111-32  | Human PPARα Reporter Assay System  
                         3x 32 assays in 8-well strips (96-well plate 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

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
</table>
| IB00131-32P | PANEL_Human PPARγ, PPARα and PPARδ Reporter Assays  
                         32 assays each in 8-well strips (96-well plate format) |

#### MOUSE PPARα Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
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</thead>
</table>
| M00111-32   | Mouse PPARα Reporter Assay System  
                         3x 32 assays in 8-well strips (96-well plate format) |
| M00111     | Mouse PPARα Reporter Assay System  
                         1x 96-well format assay |
| M00112     | Mouse PPARα Reporter Assay System  
                         1x 384-well format assays |

#### RAT PPARα Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
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</thead>
</table>
| R00111-32   | Rat PPARα Reporter Assay System  
                         3x 32 assays in 8-well strips (96-well plate format) |
| R00111     | Rat PPARα Reporter Assay System  
                         1x 96-well format assay |
| R00112     | Rat PPARα Reporter Assay System  
                         1x 384-well format assays |

Please refer to INDIGO Biosciences website for updated product offerings.

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### VI. Limited Use Disclosures

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APPENDIX 1a for tip-based dispensing. Example scheme for the serial dilution of the reference agonist GW590735 into CSM to generate 2x-concentrated treatment media. A tip-based instrument is used to dispense 15 µl / well into an assay plate that has been pre-dispensed with 15 µl / well of PPARα Reporter Cells suspension.
APPENDIX 1b for acoustic dispensing. Example scheme for the serial dilution of the reference agonist GW590735 into DMSO to generate 1,000x-concentrated stocks. 30 nl / well are pre-dispensed into an empty assay plate using an acoustic transfer device.