Human Peroxisome Proliferator-Activated Receptor Delta
(NR1C2, PPARD, PPARδ, PPARβ)

Reporter Assay System

96-well Format Assays
Product # IB00121

Technical Manual
(version 7.1)

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Human PPARδ Reporter Assay System
96-well Format Assays

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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 Delta (NR1C2), a ligand-dependent transcription factor commonly referred to as PPARD or PPARδ.

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

PPARδ 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 the pre-incubation of reporter cells prior to assay setup.

INDIGO Bioscience’s Nuclear Receptor Reporter Assays are all-inclusive cell-based assay systems. In addition to PPARδ 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 reporter assay systems 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).

INDIGO’s Nuclear Receptor Reporter Assay Systems 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 •

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using Compound Screening Medium (CSM), as described in Step 2 of the Assay Protocol. This method avoids the 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; 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</td>
<td>9.6 ml / plate</td>
<td>~ 2.4 ml</td>
</tr>
<tr>
<td>(12 ml, prepared from kit components)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDR</td>
<td>9.6 ml / plate</td>
<td>~ 2.4 ml</td>
</tr>
<tr>
<td>(12 ml, prepared from kit components)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

• Assay Scheme •

**Figure 1.** Assay workflow. **In brief,** Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user’s test compounds. Following 22-24 hr incubation, treatment media are discarded and prepared Luciferase Detection Reagent (LDR) is added. Light emission from each assay well is quantified using a plate-reading luminometer.
- Assay Performance -

**Human PPARδ Assays: Agonist dose-responses**

![Graph](image.png)

- **GW0742**
  - EC$_{50}$ = 0.41 nM
- **GW501516**
  - EC$_{50}$ = 0.61 nM
- **L-165041**
  - EC$_{50}$ = 70 nM
- **Mock Reporter Cells**
  - treated with GW0742

**Figure 2. Agonist dose-response of the PPARδ Assay.**

Validation of the PPARδ Assay was performed using manual dispensing of the reference agonists GW0742 (provided), GW501516 and L-165041 (Tocris), and following the protocol described 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 were specially prepared to contain only the luciferase reporter vector (mock reporter cells are not provided with assay kits). Final assay concentrations of agonist treatment media ranged between 40 $\mu$M and 10 pM, and included a 'no-treatment' control ($n \geq 6$ / treatment; highest [DMSO] $\leq$ 0.1% f.c. APPENDIX I describes an abbreviated 8-point dilution scheme that we find suitable for GW0742.) Mock Reporter Cells were identically treated with GW0742. Luminescence was quantified using a GloMax-Multi+ plate-reading luminometer (Promega Corp.). Average Relative Light Units (RLU) and their respective values of Standard Deviation (SD), Coefficient of Variation (CV), and Signal-to-Background (S/B) were determined for each treatment concentration. Z’ values were calculated as described by Zhang, et al. (1999)$^1$. Non-linear regression analyses were performed and EC$_{50}$ values determined using GraphPad Prism software.

**RESULTS:** PPARδ reporter cells treated with 625 nM GW0742 yielded an average RLU value with CV = 6.0%, S/B ~ 430, and a corresponding Z’ = 0.82. Mock reporter cells treated with GW0742 demonstrate no significant background luminescence ($\leq$ 1% that of the reporter cells at EC$_{Max}$). Thus, luminescence results through ligand-dependent activation of human PPARδ expressed in these reporter cells.


\[ Z' = 1 - [3 \times (SD_{Control} + SD_{Background}) / (RLU_{Control} - RLU_{Background})] \]
Figure 3. Antagonist dose-response analyses of Human PPARδ performed in combination with the INDIGO Live Cell Multiplex Assay.

(a.) PPARδ antagonist assays were performed using GSK0660 and GSK3787 (Tocris).

(b.) To confirm that the observed drop in RLU values resulted from receptor inhibition, and not induced cell death, the relative numbers of live cells in each assay well were determined at the end of the treatment period using INDIGO's Live Cell Multiplex (LCM) Assay (#LCM-01).

Final assay concentration of the agonist GW0742 was 1 nM (approximating EC₇₅), and concentrations of the respective antagonists ranged between 10 µM and 10 pM (n ≥ 6 per treatment; highest [DMSO] ≤ 0.15% f.c.). Included were cells treated with 3.0 µM Staurosporine as a positive control for cytotoxic response. Assay plates were incubated for 23 hrs, then processed according to the LCM Assay protocol to quantify relative numbers of live cells per treatment condition. Plates were then further processed to quantify PPARδ activity for each treatment condition. Averaged RFU values from each antagonist treatment group were normalized to the average RFU value of “no antagonist treatment” assay wells, which corresponds to 100% Live Cells in the LCM assay.

Results: GSK0660 and GSK3787 treatments both caused dose-dependent reductions in RLU values, down to “background” levels. However, the LCM Assay reveals no decrease in the numbers of live cells per assay well, up to the maximum treatment concentrations of 10 µM. Hence, the observed reductions in RLU values can be attributed to the inhibition of PPARδ activity by the treatment compounds, and not to induced cell death.

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 Human PPARδ Reporter Assay System contains materials to perform assays in a single 96-well assay plate.

The aliquot of PPARδ 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.

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>PPARδ 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 10.5 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>GW0742, 1.0 mM (in DMSO) (reference agonist for PPARδ)</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>
<tr>
<td>(white, sterile, cell-culture ready)</td>
<td></td>
<td></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, sterile, 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 1-8** are performed on **Day 1**, requiring less than 2 hours to complete. **Steps 9-14** 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 PPARδ Reporter Assay System kit includes a 1.0 mM stock solution of **GW0742**, an agonist of PPARδ that may be used to setup antagonist-mode assays. 3.3 nM GW0742 typically approximates EC$_{75}$ in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

We find that 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 following protocol. Note that, in **Step 6**, 100 µl of treatment media is combined with 100 µl of pre-dispensed [Reported Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.

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**DAY 1 Assay Protocol:** All steps must be performed using 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** (first see **Note 5.3**): Prepare Test Compound treatment media for **Agonist-** or **Antagonist-mode** screens.

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

Note that, in **Step 6**, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl 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). Plan dilution volumes carefully; this kit provides 35 ml of CSM.

**Preparing the positive control:** This PPARδ Reporter Assay System kit includes a 1.0 mM stock solution of the reference agonist **GW0742**. The following 8-point treatment series, with concentrations presented in 3-fold decrements, provides a suitable dose-response: 90, 30, 10, 3.33, 1.11, 0.370, 0.123, and 0.0411 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

Note that, in **Step 6**, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl 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 schemes carefully. This assay kit provides 35 ml of CSM.

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

   **Second**, retrieve **Reporter Cells** from -80°C storage. Perform a **rapid thaw** of the frozen cells by transferring a 10 ml volume of 37°C CRM into the 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 12 ml.

4.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface of the tube with a 70% alcohol swab.
5.)  

a. **Agonist-mode assays.** Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into each well of the assay plate.

   ~ or ~

b. **Antagonist-mode assays.** Gently invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain an homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "A word about antagonist-mode assay setup", pg. 8). Dispense 100 µl of cell suspension into each well of the assay plate.

**NOTE 5.1:** 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.

**NOTE 5.2:** 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 (100 µl/well) into a clear 96-well cell culture treated assay plate, followed by 100 µl/well of CSM. Incubated overnight in identical manner to those reporter cells contained in the white assay plate.

**NOTE 5.3:** For logistical reasons, some users find it more convenient to first plate the reporter cells and then prepare their test compound dilutions. That strategy works equally well. Once plated, cells may be placed in an incubator for up to 3 hours before proceeding to **Step 6.**

6.) Dispense 100 µl of 2x-concentration treatment media into appropriate assay wells.

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 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 septic technique, and may be performed on a bench top.

9.) 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.

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.) **Immediately before proceeding to Step 12,** 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.

12.) Following 22 - 24 hours of incubation discard all 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.

13.) 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. Do not shake the assay plate during this period.

14.) Quantify luminescence.


## V. Related Products

### Rat PPARδ Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>R00121-32</td>
<td>Rat PPARδ Reporter Assay System 3x 32 assays in 96-well format</td>
</tr>
<tr>
<td>R00121</td>
<td>Rat PPARδ Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>R00122</td>
<td>Rat PPARδ Reporter Assay System 1x 384-well format assays</td>
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### Mouse PPARδ Assay Products

<table>
<thead>
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<th>Product No.</th>
<th>Product Descriptions</th>
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<tbody>
<tr>
<td>M00121-32</td>
<td>Mouse PPARδ Reporter Assay System 3x 32 assays in 96-well format</td>
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<tr>
<td>M00121</td>
<td>Mouse PPARδ Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>M00122</td>
<td>Mouse PPARδ Reporter Assay System 1x 384-well format assays</td>
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### Human PPARδ Assay Products

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<th>Product Descriptions</th>
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<tbody>
<tr>
<td>IB00121-32</td>
<td>Human PPARδ Reporter Assay System 3x 32 assays in 96-well format</td>
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<tr>
<td>IB00121</td>
<td>Human PPARδ Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB00122</td>
<td>Human PPARδ Reporter Assay System 1x 384-well format assays</td>
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</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 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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</table>
LIVE Cell Multiplex (LCM) Assay

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
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<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.

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APPENDIX 1

Example scheme for the serial dilution of GW0742 reference agonist, and the setup of a PPARδ dose-response assay.

*For convenience, serial dilutions may be made directly in a dual function solution basin (Heathrow Scientific) or a deep 96-well plate.