Human Peroxisome Proliferator-Activated Receptor Reporter Assays

PANEL

PPARα, PPARδ, PPARγ

32 Assays each in 96-well Format
Product #IB00131-32P

Technical Manual
(version 7.1)

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Human PPAR Reporter Assays PANEL
PPARα, PPARδ, PPARγ
32 Assays each in 96-well Format

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I. Description

- The Assay System -

INDIGO's PANEL of PPAR Reporter Assays utilizes non-human mammalian cells engineered to express Human Peroxisome Proliferator-Activated Receptors: PPARα (NR1C1), PPARδ (NR1C2), or PPARγ (NR1C3), all ligand-dependent transcription factors that are commonly referred to as PPARα, PPARδ and PPARγ.

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

PPAR 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 PPAR 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 Mg⁺²-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.
**Assay Scheme**

Figure 1. Assay workflow. *In brief*, PPAR Reporter Cells are dispensed into 32 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 sample well is quantified using a plate-reading luminometer.

**Assay Performance**

Figures 2, 3, and 4 present performance data for the PPARα, PPARδ, and PPARγ assays. 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 agonist, as noted in respective figures (mock reporter cells are not provided with assay kits). For each assay, 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.

$$Z' = 1 - \frac{3 \times (SD_{\text{Control}} + SD_{\text{Background}})}{(RLU_{\text{Control}} - RLU_{\text{Background}})}$$

Figure 2. *Agonist dose-response analyses of Human PPARα.* Analyses of PPARα Reporter Cells using GW590735 (provided), GW7647 and WY14643 (Tocris). Final assay concentrations varied between 40 µM and 0.010 nM and included a 'no-treatment' control (n ≥ 6; highest [DMSO] ≤ 0.1% f.c.). Appendix 1 describes an abbreviated 8-point dilution scheme for GW590735). Mock reporter cells demonstrate no significant background luminescence (≤ 0.1% that of the reporter cells at EC₅₀). Thus, luminescence results strictly through ligand-activation of PPARα expressed in these reporter cells. Z’ scores confirm the robust performance of this PPARα Assay.

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Figure 3a. Agonist dose-response analyses of Human PPARδ.
Validation of the PPARδ Assay was performed using manual dispensing of the reference agonists GW0742 (provided), GW501516 and L-165041 (Tocris). Final assay concentrations of agonist treatment media ranged between 40 µM and 10 pM, and included a 'no-treatment' control (n ≥ 6 / treatment; highest [DMSO] ≤ 0.1% f.c. Appendix 2 describes an abbreviated 8-point dilution scheme that we find suitable for GW0742.) Mock Reporter Cells were identically treated with GW0742.

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 (≤ 1% that of the reporter cells at EC₅₀). Thus, luminescence results through ligand-dependent activation of human PPARδ expressed in these reporter cells.
(a.) Human PPARδ Antagonist Assays

![Antagonist dose-response analyses of Human PPARδ performed in combination with the INDIGO Live Cell Multiplex Assay.](image)

(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 EC_{75}), 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.

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.
Validation of the PPARγ Assay was performed using manual dispensing and following the protocol described in this Technical Manual, using the reference agonists Rosiglitazone (provided), Troglitazone (Tocris) and Ciglitazone (Tocris). PPARγ Reporter Cells and Mock reporter cells were identically treated with Rosiglitazone, as described in Appendix 3.

PPARγ reporter cells treated with 2,500 nM Rosiglitazone yielded an average RLU value with CV=7%, S/B = 162 and a corresponding $Z'$ = 0.78. Similarly treated mock reporter cells demonstrate no significant background luminescence ($\leq 0.05\%$ that of EC$_{Max}$). Thus, luminescence results strictly through ligand-activation of the PPARγ expressed in these reporter cells.
Figure 4b. **Antagonist dose-response analyses of Human PPARγ performed in combination with the INDIGO Live Cell Multiplex Assay.**

Antagonist assays were performed using T0070907 (Tocris), and GW9662 (Tocris). To confirm that the observed drop in RLU values resulted from receptor inhibition, as opposed to induced cell death, the relative numbers of live cells in each assay well were determined using INDIGO's Live Cell Multiplex (LCM) Assay (#LCM-01). Final assay concentrations of the respective antagonists ranged between 10 µM and 10 pM, including a 'no antagonist' control (n ≥ 6 per treatment; highest [DMSO] ≤ 0.15% f.c.). Each treatment also contained 220 nM (approximating EC50) Rosiglitazone as challenge agonist. Assay plates were incubated for 22 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 PPARγ 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.

T0070907 and GW9662 both caused dose-dependent reduction in RLU values. The LCM Assay reveals no significant variance in the numbers of live cells per assay well, up to the maximum treatment concentration of 10 µM. Hence, the observed reduction in RLU values can be attributed to dose-dependent inhibition of PPARγ activity, and not to cell death.
II. Product Components & Storage Conditions

This Human PPAR Reporter Assays PANEL contains materials to perform 32 PPARα assays, 32 PPARδ assays, and 32 PPARγ assays, all in a single 96-well plate format. All reagents are supplied with sufficient extra volume to accommodate the needs of performing 3 individual groups of assays.

The individual aliquots of PPAR Reporter Cells and Detection Substrate and Detection Buffer are provided as single-use reagents. Once thawed, reporter cells can NOT be refrozen 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.

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<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
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<tbody>
<tr>
<td>• PPARα Reporter Cells</td>
<td>1 x 0.60 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• PPARδ Reporter Cells</td>
<td>1 x 0.60 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• PPARγ Reporter Cells</td>
<td>1 x 0.60 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Cell Recovery Media (CRM)</td>
<td>1 x 10.5 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Compound Screening Media (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• PPARα reference agonist:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GW590735, 10 mM (in DMSO)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• PPARδ reference agonist:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GW0742, 1.0 mM (in DMSO)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• PPARγ reference agonist:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosiglitazone, 10 mM (in DMSO)</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>
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<tr>
<td>• 96-well format plate frame</td>
<td>1</td>
<td>ambient</td>
</tr>
<tr>
<td>• snap-in, 8-well strips</td>
<td>12</td>
<td>ambient</td>
</tr>
<tr>
<td>(white, sterile, cell culture treated)</td>
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</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 or deep-well plates, or appropriate similar vessel for generating serial dilutions of test & reference compound(s).
- antagonist reference compounds (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 9-15** 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 EC₅₀ – EC₈₅) of a known agonist AND the test compound(s) to be evaluated for antagonist 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.

This PPAR Assay Panel kit provides a commonly used reference agonist for each PPAR assay; they may be used effectively to setup respective receptor inhibition assays.

- **PPARα**: GW590735 is provided as a 10 mM stock in DMSO; it may be used as an agonist of PPARα (Figure 2A) to set up antagonist screens. 33.3 nM GW590735 typically approximates EC₇₀ in this reporter assay.

- **PPARγ**: GW0742 is provided as a 1.0 mM stock in DMSO; it may be used as an agonist of PPARδ (Figure 3A) to set up antagonist screens. 3.3 nM GW0742 typically approximates EC₇₀ in this reporter assay.

- **PPARγ**: Rosiglitazone is provided as a 10 mM stock in DMSO; it may be used as an agonist of PPARγ (Figure 4A) to set up antagonist screens. 300 nM Rosiglitazone typically approximates EC₇₀ in this reporter assay.

**Note**: In **Step 6**, 100 µl of treatment media is combined with 100 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist.
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.

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). Use CSM to prepare the appropriate dilution series. Manage dilution volumes carefully. This assay kit provides 35 ml of CSM.

Preparin the positive control: This PPAR Assay Panel kit provides a commonly used reference agonist for each PPAR assay.

- **PPARα.** Agonist GW590735 is provided as a 10 mM stock in DMSO. We find the PPARα assay exhibits a complete dose-response to GW590735 using an assay concentration range of: 300, 100, 33.3, 11.1, 3.70, 1.23, 0.412 and 0 nanoMolar (nM; 10⁻⁹ Molar), as depicted in Figure 2A.

- **PPARδ.** Agonist GW0742 is provided as a 1.0 mM stock in DMSO. We find the PPARδ assay exhibits a complete dose-response to GW0742 using an assay concentration range of: 90, 30, 1.0, 3.33, 1.11, 0.370, 0.123, 0.412 and 0 nM, as depicted in Figure 3A.

- **PPARγ.** Agonist Rosiglitazone is provided as a 10 mM stock in DMSO. We find the PPARγ assay exhibits a complete dose-response to Rosiglitazone using an assay concentration range of: 2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 0 nM, as depicted in Figure 4A.

3.) Rapid Thaw of the Reporter Cells: 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. Perform a rapid thaw of the frozen cells by transferring a 3.0 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 3.6 ml.

Third, work in the cell culture hood to carefully mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

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

5.) a. Agonist-mode assays. Invert the tube of PPAR Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into respective strip-wells 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 3.6 ml bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to “A word about agonist-mode assay setup”, pg.10). Dispense 100 µl of cell suspension into respective strip-wells of the assay plate.

**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.
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.

9.) 30 minutes before intending to quantify ERβ activity, remove **Detection Substrate** from the refrigerator and place them in a low-light area so that it may equilibrate to room temperature. Gently invert the tube several times to ensure an homogenous solution.
   
   **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:** 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.

12.) After 22-24 hours of incubation, remove media contents from each well.
   
   **NOTE:** Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. 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. 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.

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.

**DAY 2 Assay Protocol:** Subsequent manipulations do not require special regard for aseptic technique, and may be performed on a bench top.
V. Related Products

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<th>Product Descriptions</th>
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<td>IB00111-32</td>
<td>Human PPARα Reporter Assay System 3x 32 assays in 96-well format</td>
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<td>IB00111</td>
<td>Human PPARα Reporter Assay System 1x 96-well format assay</td>
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<td>IB00112</td>
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### PPARδ Assay Products

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### PPARγ Assay Products

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<td>IB00102</td>
<td>Human PPARγ Reporter Assay System 1x 384-well format assays</td>
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Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

### Panel of PPAR Assays

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Mouse/Rat PPARγ Assay Products

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<td>MR00102</td>
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Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

Panel of Mouse PPAR Assay Products

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LIVE Cell Multiplex (LCM) Assay

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<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>
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<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>
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<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>
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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.

APPENDIX 2
Example scheme for the serial dilution of GW0742 reference agonist, and the setup of a PPARδ dose-response assay.
APPENDIX 3

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

[Diagram of serial dilution and dose-response assay setup]