Human Peroxisome Proliferator-Activated Receptor Gamma
(NR1C3, PPARG, PPARγ)

Reporter Assay System

3x 32 Assays in 96-well Format
Product #IB00101-32

Technical Manual
(version 7.1b)

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Human PPARγ Reporter Assay System
3x 32 Assays in 96-well Format

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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 Gamma (NR1C3), a ligand-dependent transcription factor commonly referred to as PPARγ.

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

PPARγ 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 assays are all-inclusive cell-based assay. In addition to PPARγ 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 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 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 assay 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, 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 PPARγ Assay: Agonist dose-response assays

![Graph showing dose-response curves for Rosiglitazone, Troglitazone, Ciglitazone, and Mock Reporter Cells treated with Rosiglitazone.](image)

**Figure 2a. Agonist dose-response analyses of the Human PPARγ Assay.**

Validation of the PPARγ Assay was performed using manual dispensing and following the protocol described in this Technical Manual, using the reference agonists Rosiglitazone (Cayman Chemical), Troglitazone (Tocris) and Ciglitazone (Tocris). In addition, to assess the level of background signal contributed by non-specific factor(s) 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). PPARγ Reporter Cells and Mock reporter cells were identically treated with Rosiglitazone, as described in Appendix 1. Luminescence was quantified using a GloMax-Multi+ plate-reading luminometer (Promega Corp.). Values of average Relative Light Units (RLU; average of n ≥ 6), respective standard deviation (SD), Signal-to-Background (S/B) and Coefficient of Variation (CV) were determined. Z’ values were calculated as described by Zhang, et al. (1999).

Non-linear regression analyses were performed and EC50 values determined using GraphPad Prism software.

RESULTS: 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 (≤ 0.05% that of ECMax). Thus, luminescence results strictly through ligand-activation of the PPARγ expressed in these reporter cells.

Z’ = 1 - [3*(SD\text{Control} + SD\text{Background}) / (RLU\text{Control} - RLU\text{Background})]

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**Figure 2b.** *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.

**Results:** 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.

**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 PPARγ assay 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, reagents may be combined to perform either 64 or 96 assays.

The individual aliquots of Reporter Cells are provided as single-use reagents. 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>▪ PPARγ Reporter Cells</td>
<td>3 x 0.60 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>▪ Rosiglitazone, 10 mM (in DMSO)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>(reference agonist for PPARγ)</td>
<td></td>
<td></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</td>
<td>12</td>
<td>ambient</td>
</tr>
</tbody>
</table>

(white, sterile, cell-culture ready)

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**
- container of dry ice (see Step 2)
- 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 PPARγ assay kit includes a 10 mM stock solution of Rosiglitazone, an agonist of PPARγ that may be used to setup antagonist-mode assays. 225 nM Rosiglitazone typically approximates EC$_{50}$ in this assay. Hence, it presents a suitable 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 [Reporter 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.

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

Preparing the positive control: This PPARγ assay kit includes a 10 mM stock solution of Rosiglitazone, a reference agonist of PPARγ. The following 7-point treatment series, with concentrations presented in 4-fold decrements, provides a suitable dose-response: 10000, 2500, 625, 156, 39.1, 9.77, and 2.44 nM, and including a 'no treatment' control. APPENDIX 1 provides an example for generating such a dilution series.
3.) **Rapid Thaw of the Reporter Cells:** *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 and place them directly into dry ice to transport the cells to the laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, and 3 tubes for 96 assay wells. When ready to begin, place the tube of reporter cells into a rack and, **without delay,** perform a rapid thaw of the frozen cells by transferring a 3.0 ml volume of 37°C 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. If only one tube of reporter cells is thawed (32 assays), 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, then transfer it into the cell culture hood.

5.) **a. Agonist-mode assays.** Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain a 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 a 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:* 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.
9.) 30 minutes before intending to quantify receptor 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 a 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.
V. Related Products

### Human PPARγ Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Description</th>
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<tbody>
<tr>
<td>IB00101-32</td>
<td>Human PPARγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)</td>
</tr>
<tr>
<td>IB00101</td>
<td>Human PPARγ Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB00102</td>
<td>Human PPARγ Reporter Assay System 1x 384-well format assays</td>
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### Panel of Human PPAR Assays

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

### Mouse/Rat PPARγ Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Description</th>
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<tr>
<td>MR00101-32</td>
<td>Mouse/Rat PPARγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)</td>
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<tr>
<td>MR00101</td>
<td>Mouse/Rat PPARγ Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>MR00102</td>
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</tr>
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</table>

### Zebrafish PPARγ Assay Products

<table>
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<th>Product No.</th>
<th>Product Description</th>
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<tbody>
<tr>
<td>Z00101-32</td>
<td>Zebrafish PPARγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)</td>
</tr>
<tr>
<td>Z00101</td>
<td>Zebrafish PPARγ Reporter Assay System 1x 96-well format assay</td>
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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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<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, diagnostic, or contact use in humans or animals.

“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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Example scheme for the serial dilution of Rosiglitazone reference agonist, and the setup of a PPARγ dose-response assay.
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96-well Format Assays
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• 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 12 ml</td>
<td>100 µl / well 9.6 ml / plate</td>
<td>~ 2.4 ml</td>
</tr>
<tr>
<td>(prepared from kit components)</td>
<td></td>
<td></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. 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.
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\[ Z' = 1 - \frac{3 \times (SD_{Control} + SD_{Background})}{RLU_{Control} - RLU_{Background}} \]

Figure 2b. **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.

**Results:** 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.

**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 PPAR\(\gamma\) Reporter assay contains materials to perform assays in a single 96-well assay plate.

The aliquot of PPAR\(\gamma\) 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 Component</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPAR(\gamma) 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>Rosiglitazone, 10 mM (in DMSO) (reference agonist for PPAR(\gamma))</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 (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**
- container of dry ice (used in Step 3)
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO\(_2\) 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 PPARγ assay kit includes a 10 mM stock solution of Rosiglitazone, an agonist of PPARγ that may be used to setup antagonist-mode assays. 225 nM Rosiglitazone typically approximates EC$_{50}$ in this assay. Hence, it presents a suitable 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 [Reporter 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.

---

**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 PPARγ assay kit includes a 10 mM stock solution of Rosiglitazone, a reference agonist of PPARγ. The following 7-point treatment series, with concentrations presented in 4-fold decrements, provides a suitable dose-response: 10000, 2500, 625, 156, 39.1, 9.77, and 2.44 nM (final assay concentrations), and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

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

*Second, retrieve the tube of PPARγ Reporter Cells from -80°C storage, place it directly into dry ice and transport the cells to the laminar flow hood. When ready to begin, place the tube of reporter cells into a rack and, without delay, perform a rapid thaw of the cells by dispensing a 10 ml volume of 37°C CRM directly 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 12 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.
5.) **a. Agonist-mode assays.** Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain a 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 a 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:** 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% CO$_2$ 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 aseptic 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

### Human PPARγ Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00101-32</td>
<td>Human PPARγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)</td>
</tr>
<tr>
<td>IB00101</td>
<td>Human PPARγ Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB00102</td>
<td>Human PPARγ Reporter Assay System 1x 384-well format assays</td>
</tr>
</tbody>
</table>

### 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>
</tbody>
</table>

### Mouse/Rat PPARγ Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR00101-32</td>
<td>Mouse/Rat PPARγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)</td>
</tr>
<tr>
<td>MR00101</td>
<td>Mouse/Rat PPARγ Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>MR00102</td>
<td>Mouse/Rat PPARγ Reporter Assay System 1x 384-well format assays</td>
</tr>
</tbody>
</table>

### Zebrafish PPARγ Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
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</thead>
<tbody>
<tr>
<td>Z00101-32</td>
<td>Zebrafish PPARγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)</td>
</tr>
<tr>
<td>Z00101</td>
<td>Zebrafish PPARγ Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>Product No.</td>
<td>Product Descriptions</td>
</tr>
<tr>
<td>------------</td>
<td>---------------------------------------------------------------------------------------</td>
</tr>
<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, diagnostic, or contact use in humans or animals.

“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 Rosiglitazone reference agonist and the setup of a PPARγ dose-response assay.
Human Peroxisome Proliferator-Activated Receptor Gamma (NR1C3, PPARG, PPARγ)

Reporter Assay System

384-well Format Assays
Product # IB00102

Technical Manual
(version 8.0)

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I. Description

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▪ Assay Scheme........................................................................5
▪ Assay Performance...............................................................5

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IV. Assay Protocol

▪ A word about Antagonist-mode assay setup............................7
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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 Gamma (NR1C3), a ligand-dependent transcription factor commonly referred to as PPARG or PPARγ.

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

PPARγ 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's assay kits provide the convenience of an all-inclusive cell-based assay system. In addition to PPARγ Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples, as well as the reference agonist Rosiglitazone, 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 Mg²⁺-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 <strong>tip dispensing</strong> 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 <strong>acoustic dispensing</strong> 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><strong>Detection Substrate</strong> 7.8 ml</td>
<td>15 µl / well 5.8 ml / plate</td>
<td>~ 2 ml</td>
</tr>
</tbody>
</table>
**Assay Scheme**

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**

**Figure 2.** Agonist and Antagonist dose-response of Human PPARγ. Reporter Cells were treated with the reference agonists Rosiglitizone (provided), Toglitazone and Cigitazone, as well as antagonists T007097 and GW9662. Average Relative Light Units (RLU) and respective Standard Deviation (SD) values were calculated for each treatment concentration (n=4). $Z'$ was calculated as per Zhang, et al. (1999)$^1$.

Treatment concentrations were Log10 transformed and respective RLU values were normalized as Fold-Activation. Data were plotted via non-linear regression and EC$_{50}$ and IC$_{50}$ values were determined using GraphPad Prism software.


$$Z' = 1 - \frac{[3*(SD_{Reference} + SD_{Vehicle\ Bkg})]}{RLU_{Reference} - RLU_{Vehicle\ Bkg}]}$$
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>PPARγ 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>Rosiglitazone, 10 mM (in DMSO)</td>
<td>1 x 90 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>(reference agonist for PPARγ)</td>
<td></td>
<td></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</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

- 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 10 mM stock solution of **Rosiglitazone**, an agonist of PPARγ that may be used to setup antagonist-mode assays. 225 nM Rosiglitazone typically approximates EC$_{60-80}$ in this assay. Hence, it is a suitable *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-dispersed 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 10 mM stock solution of Rosiglitazone, the most commonly used reference agonist of PPARγ. The following 7-point treatment series, with concentrations presented in 4-fold decrements, provides a complete dose-response: 10000, 2500, 625, 156, 39.1, 9.77, and 2.44 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 homogeneous suspension.

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

   ~ or ~

   *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 homogeneous 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.

   ~ or ~

   *b. for Antagonist-mode assays:* Supplement a bulk volume of CSM with the challenge agonist Rosiglitazone to achieve an *EC<sub>50</sub> – EC<sub>80</sub>* concentration (refer to "A word about antagonist-mode assay setup", pg. 7). 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 PPARγ Assay Products

<table>
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<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
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<tr>
<td>IB00101-32</td>
<td>Human PPARγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)</td>
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<tr>
<td>IB00101</td>
<td>Human PPARγ Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB00102</td>
<td>Human PPARγ Reporter Assay System 1x 384-well format 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

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<th>Product Descriptions</th>
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<td>IB00131-32P</td>
<td>PANEL_Human PPARγ, PPARα and PPARδ Reporter Assay 32 assays each in 8-well strips (96-well plate format)</td>
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#### Mouse/Rat PPARγ Assay Products

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<td>Mouse/Rat PPARγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)</td>
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<td>MR00101</td>
<td>Mouse/Rat PPARγ Reporter Assay System 1x 96-well format assay</td>
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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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<td>PANEL_mrPPARγ, mPPARα and mPPARδ Reporter Assay 32 assays each in 8-well strips (96-well plate format)</td>
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</table>

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

### VI. Limited Use Disclosures

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APPENDIX 1a for tip-based dispensing. Example scheme for the serial dilution of the reference agonist Rosiglitazone 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 PPARγ Reporter Cells suspension.
**APPENDIX 1b for acoustic dispensing.** Example scheme for the serial dilution of the reference agonist Rosiglitazone into DMSO to generate 1,000x-concentrated stocks. 30 nl / well are pre-dispensed into an empty assay plate using an acoustic transfer device.

<table>
<thead>
<tr>
<th>Stepwise Dilutions</th>
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<th>Volume (µL)</th>
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<tr>
<td>1/4 x</td>
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<tr>
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