



**Human Peroxisome Proliferator-Activated  
Receptor Gamma  
(NR1C3, PPARG, PPAR $\gamma$ )**

**Reporter Assay System**

**384-well Format Assays**  
Product # IB00102

▪

**Technical Manual**  
*(version 7.1)*

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## Human PPAR $\gamma$ Reporter Assay System 384-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 Gamma** (NR1C3), a ligand-dependent transcription factor commonly referred to as PPAR $\gamma$  or **PPAR $\gamma$** .

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

PPAR $\gamma$  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 $\gamma$  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<sup>+2</sup>-dependent reaction that consumes O<sub>2</sub> and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP<sub>i</sub>, CO<sub>2</sub>, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer, and is reported in terms of Relative Light Units (RLU's).

INDIGO's Nuclear Receptor Assay Systems 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.

▪ **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.

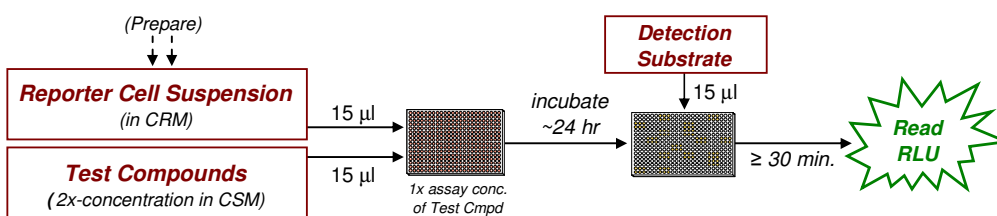
▪ **Considerations for Automated Dispensing** ▪

When processing a small number of assay plates, first carefully considered 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.

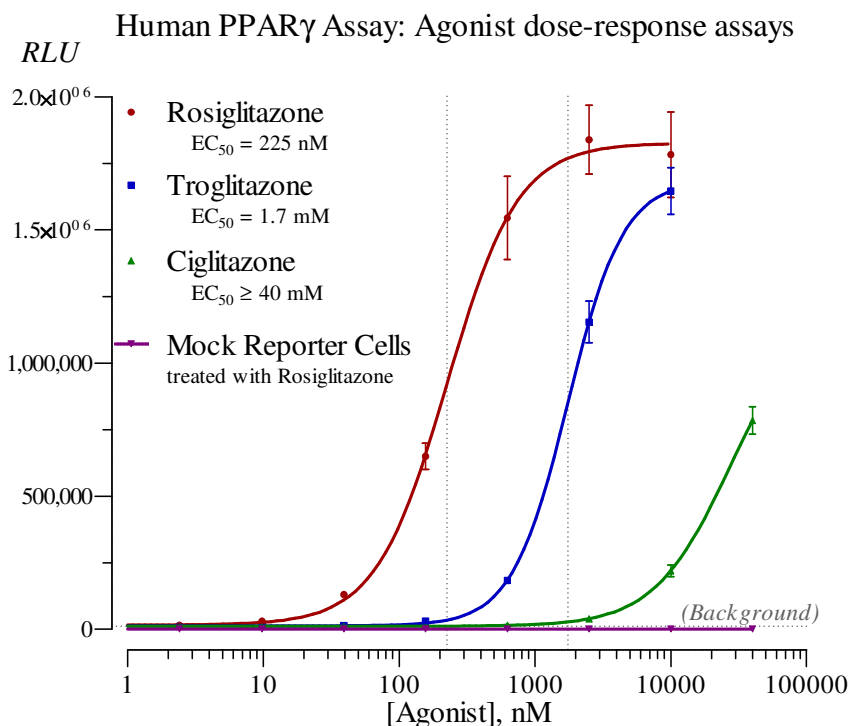
<b>Stock Reagent &amp; Volume provided</b>	<b>Volume to be Dispensed (384-well plate)</b>	<b>Excess rgt. volume available for instrument dead volume</b>
<b>Reporter Cell Suspension</b> 7.5 ml <i>(prepared from kit components)</i>	15 µl / well 5.8 ml / plate	~ 1.7 ml
<b>Detection Substrate</b> 7.8 ml	15 µl / well 5.8 ml / plate	~ 2 ml

▪ **Assay Scheme** ▪

**Figure 1.** Assay workflow. *In brief*, the prepared suspension of thawed 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 Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.



▪ Assay Performance ▪



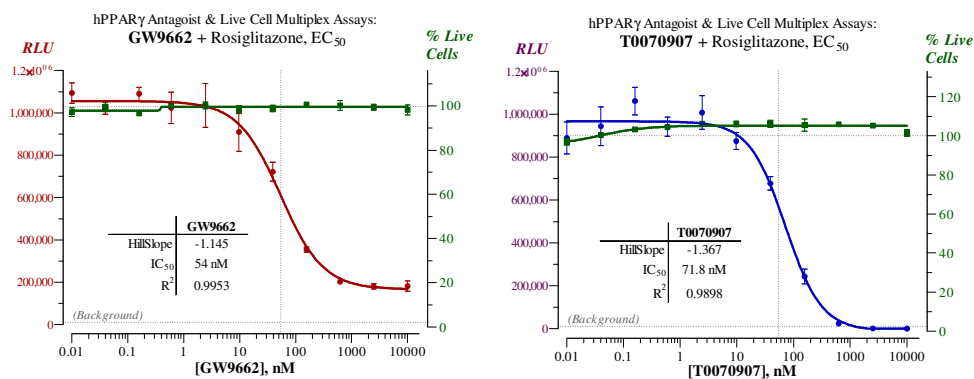
**Figure 2a. Agonist dose-response analyses of the Human PPAR $\gamma$  Assay.**

Validation of the PPAR $\gamma$  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). 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). PPAR $\gamma$  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  $n \geq 6$ ), 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)<sup>1</sup>. Non-linear regression analyses were performed and EC<sub>50</sub> values determined using GraphPad Prism software.

**RESULTS:** PPAR $\gamma$  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<sub>Max</sub>). Thus, luminescence results strictly through ligand-activation of the PPAR $\gamma$  expressed in these reporter cells.

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

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



**Figure 2b. Antagonist dose-response analyses of Human PPAR $\gamma$  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  $\mu$ M and 10 pM, including a 'no antagonist' control ( $n \geq 6$  per treatment; highest [DMSO]  $\leq 0.15\%$  *f.c.*). Each treatment also contained 220 nM (approximating EC<sub>50</sub>) 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 PPAR $\gamma$  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  $\mu$ M. Hence, the observed reduction in RLU values can be attributed to dose-dependent inhibition of PPAR $\gamma$  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 System contains materials to perform assays in a single 384-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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ PPAR $\gamma$ Reporter Cells	1 x 2.0 mL	<b>-80°C</b>
▪ Cell Recovery Medium (CRM)	1 x 6 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ Rosiglitazone, 10 mM (in DMSO) (reference agonist for PPAR $\gamma$ )	1 x 30 $\mu$ L	-20°C
▪ Detection Substrate	1 x 7.8 mL	<b>-80°C</b>
▪ 384-well assay plate (white, sterile, cell-culture ready)	1	ambient

## III. Materials to be Supplied by the User

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

### DAY 1

- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO<sub>2</sub> incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8- or 12-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media basins, sterile.
- sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), or deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- 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 9-15* 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 between EC<sub>50</sub> – EC<sub>85</sub>) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This PPAR $\gamma$  Reporter Assay System kit includes a 10 mM stock solution of **Rosiglitazone**, an agonist of PPAR $\gamma$  that may be used to setup antagonist-mode assays. 225 nM Rosiglitazone typically approximates EC<sub>50</sub> 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*, 15  $\mu$ l of treatment media is combined with 15  $\mu$ l 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 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.

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

Note that, in *Step 6*, 15  $\mu$ l of the prepared treatment media is added into assay wells that have been pre-dispensed with 15  $\mu$ l 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.

**Preparing the positive control:** This PPAR $\gamma$  Reporter Assay System kit includes a 10 mM stock solution of **Rosiglitazone**, a reference agonist of PPAR $\gamma$ . 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.) *First*, retrieve the tube of **CRM** from the 37°C water bath, sanitize the outside with a 70% ethanol swab, then place it in the cell-culture hood.

*Second*, retrieve **Reporter Cells** from -80°C storage. 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 immediately 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 and sanitize the outside surface of the tube with a 70% alcohol swab.

5.) *a. Agonist-mode assays.* Invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 15 µl of cell suspension into each well of the assay plate.

~ or ~

*b. Antagonist-mode assays.* 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 15 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 prefer to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear assay plate, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense 15 µl of 2x-concentration treatment media into appropriate assay wells.

7.) Transfer the assay plate into a 37°C, humidified 5% CO<sub>2</sub> incubator for 22 - 24 hours.

*NOTE:* Ensure a high-humidity (≥ 90%) 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.) 30 minutes before intending to quantify PPAR $\gamma$  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.) Following 22 - 24 hours of incubation add 15  $\mu$ l of **Detection Substrate** to each well of the assay plate.

*NOTE: Perform manual reagent transfers 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 may significantly degrade the accuracy and precision of the assay data. In the event of excessive bubble formation during manual processing, spin the assay plate (with lid) at *low speed* 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

<b>Human PPAR<math>\gamma</math> Assay Products</b>	
<i>Product No.</i>	<i>Product Descriptions</i>
IB00101-32	Human PPAR $\gamma$ Reporter Assay System 3x 32 assays in 96-well format
IB00101	Human PPAR $\gamma$ Reporter Assay System 1x 96-well format assay
IB00102	Human PPAR $\gamma$ Reporter Assay System 1x 384-well format assays
Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

<b>Panel of Human PPAR Assays</b>	
<i>Product No.</i>	<i>Product Description</i>
IB00131-32P	Human PPAR $\gamma$ , PPAR $\alpha$ and PPAR $\delta$ Reporter Assay PANEL 32 assays each in 1x 96-well plate

<b>Mouse/Rat PPAR<math>\gamma</math> Assay Products</b>	
<i>Product No.</i>	<i>Product Descriptions</i>
MR00101-32	Mouse/Rat PPAR $\gamma$ Reporter Assay System 3x 32 assays in 96-well format
MR00101	Mouse/Rat PPAR $\gamma$ Reporter Assay System 1x 96-well format assay
MR00102	Mouse/Rat PPAR $\gamma$ Reporter Assay System 1x 384-well format assays
Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

<b>Panel of Mouse PPAR Assay Products</b>	
<i>Product No.</i>	<i>Product Description</i>
MR00131-32P	mrPPAR $\gamma$ , mPPAR $\alpha$ and mPPAR $\delta$ Reporter Assay PANEL 32 assays each in 1x 96-well plate

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

Please refer to INDIGO Biosciences website for updated product offerings.

[www.indigobiosciences.com](http://www.indigobiosciences.com)

### ***VI. Limited Use Disclosures***

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans. Other applications of this product may require licenses from others.

The method of recombinant expression of the human PPAR $\gamma$  is a patented technology. INDIGO Biosciences, Inc. has entered into a license agreement with the assignees that mandates the following disclosure:

“Before using this product, carefully read the Patent and Licensing Information below. By using this product, you indicate your acceptance of the conditions described below.

Considerable time and money has been spent developing this product which incorporates, among other things, the PPAR-gamma protein, which is sold under certain patent rights including US Patent No. 6,200,802. INDIGO Biosciences recognizes the importance of providing a product using this technology for ongoing research in the area of drug discovery and molecular and cellular biology, and we therefore want to make the kit available as widely as possible to the scientific community. However, our rights to the product as covered by the aforementioned patent rights are limited to a license for research use only from the Inventors to the end user provided the product has been purchased directly from INDIGO Biosciences.

The end user agrees that it will not sell, transfer, disclose or otherwise provide access to this product to any person or entity, other than its employees. The end user acknowledges that it does not have the right to authorize any third party to use or sell any products covered under the aforementioned product or derivatives thereof.

The end user agrees that if any useful chemicals are identified using this product in its research, any commercial use thereof requires a license for such commercial use from the representative of the inventors at 191 N. Wacker Drive, Suite 1800, Chicago Illinois 60606, (312)521-2778. If you are in doubt as to whether your use of the molecules or chemicals derived from use of this product would require a commercial license, please discuss this matter with Jack Shore, Esq. at the provided address or phone number.”

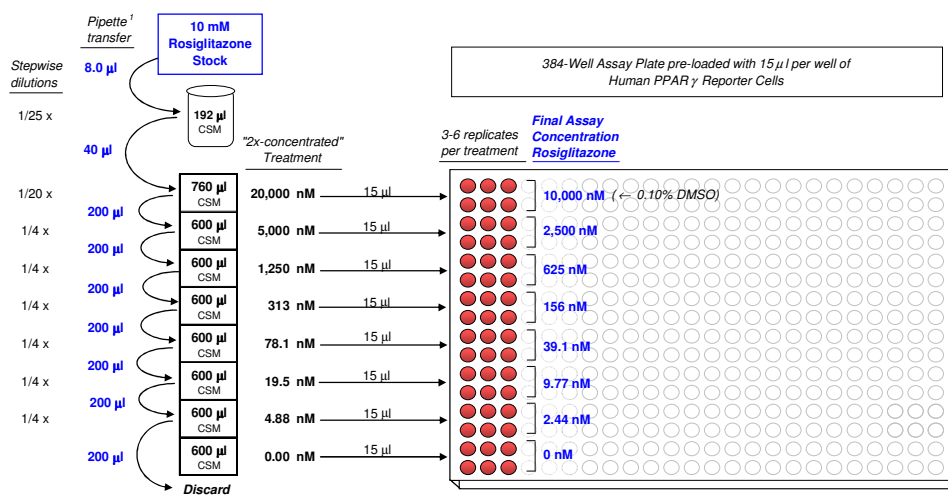
“CryoMite” is a Trademark <sup>TM</sup> of INDIGO Biosciences, Inc. (State College, PA, USA)

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 PPAR $\gamma$  dose-response assay.



<sup>1</sup> For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.