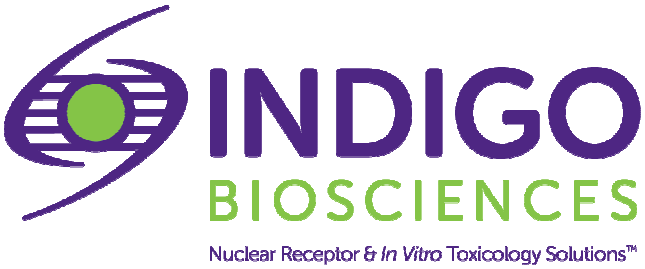

Rat Peroxisome Proliferator-Activated Receptor Delta

(nr1c2, pparD, rPPARδ, rPPARβ)

Reporter Assay System

3x 32 Assays in 96-well Format
Product # R00121-32

▪

Technical Manual
(version 7.1b)

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INDIGO BIOSCIENCES
Nuclear Receptor & In Vitro Toxicology Solutions™

Rat PPARδ Reporter Assay System
3x 32 Assays in 96-well Format

I. Description

▪ The Assay System ▪

This nuclear receptor assay utilizes proprietary non-human cells engineered to provide constitutive, high-level expression of the **Rat Peroxisome Proliferator-Activated Receptor Delta** (nr1c2), a ligand-dependent transcription factor commonly referred to as pparD or **rat PPARδ**.

INDIGO's 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 rat PPARδ activity. The principal application of this reporter assay is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against rat PPARδ.

Rat PPARδ Reporter Cells are prepared using INDIGO's proprietary **CryoMite™** process. This cryo-preservation method yields exceptional cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for cumbersome intermediate treatment steps such as spin-and-rinse of cells, viability determinations, cell titer adjustments, or the pre-incubation of reporter cells prior to assay setup.

INDIGO Bioscience's Nuclear Receptor Reporter Assays are all-inclusive cell-based assay kits. In addition to rat PPARδ Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, a reference agonist, Luciferase Detection Reagent, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's nuclear receptor reporter assays capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology.

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (*Photinus pyralis*). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg^{+2}-dependent reaction that consumes O_2 and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, CO_2, 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 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.

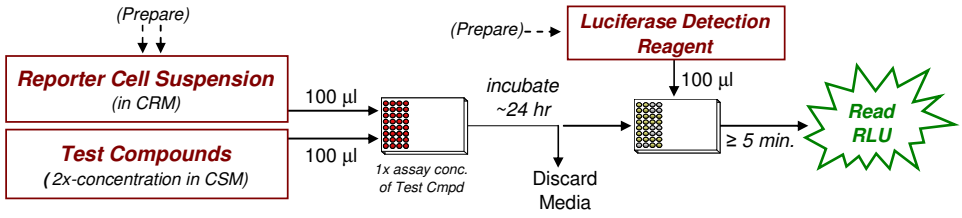
▪ Preparation of Test Compounds ▪

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%.

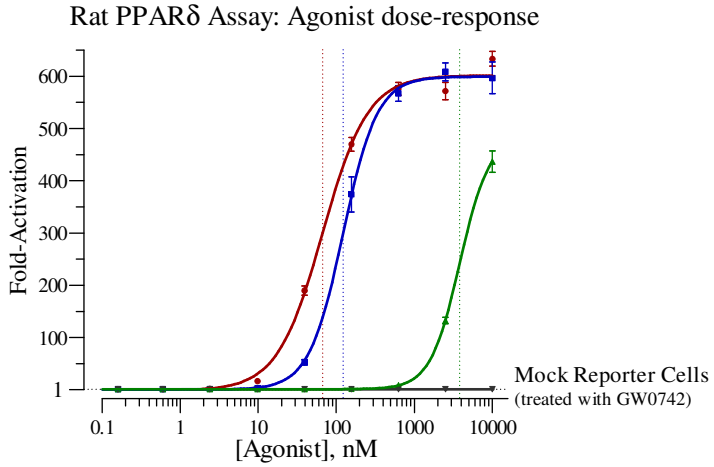
NOTE: CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. 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** ▪



• GW0742
EC_{50} = 67 nM
Hill slope = 1.501
R^2 = 0.9973

at 2.5 µM:
S/B = 570
%CV = 2.9
z' = 0.91

▪ GW501516
EC_{50} = 120 nM
Hill slope = 1.995
R^2 = 0.9997

at 2.5 µM:
S/B = 600
%CV = 3.0
z' = 0.91

▴ L-165,041
est'd EC_{50} ~ 3.8 µM
Hill slope = 2.313
R^2 = 1.000

at 10 µM:
S/B = 440
%CV = 4.7
z' = 0.86

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

Dose-response analyses of rPPARδ Reporter Cells were performed using GW0742 (provided), GW501516 (Enzo) and L-165041 (Tocris). 10 mM master stocks were prepared in DMSO for each agonist. Final assay concentrations were prepared by serial dilution in 4-fold decrements, ranging between 10 µM and 0.15 nM. The highest assay concentration of residual DMSO was 0.1%, which has no effect on assay performance. To assess the level of background signal contributed by non-specific factors that cause gratuitous activation of the luciferase reporter gene, "mock" reporter cells were treated with GW0742 (mock reporter cells, which contain only the luciferase reporter vector, are not provided with assay kits). Treatment media were removed after 24 hr and LDR was applied. 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 ($n \geq 6$). Fold-Activation (*i.e.*, Signal-to-background; S/B) and Z' values were calculated as described by Zhang, *et al.* (1999)[1]. Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software.

RESULTS: The high S/B and Z' values, low background and %CV values, and the absence of non-specific reporter activity confirm the robust performance of this rPPARδ Reporter Assay.

[1] 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.

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

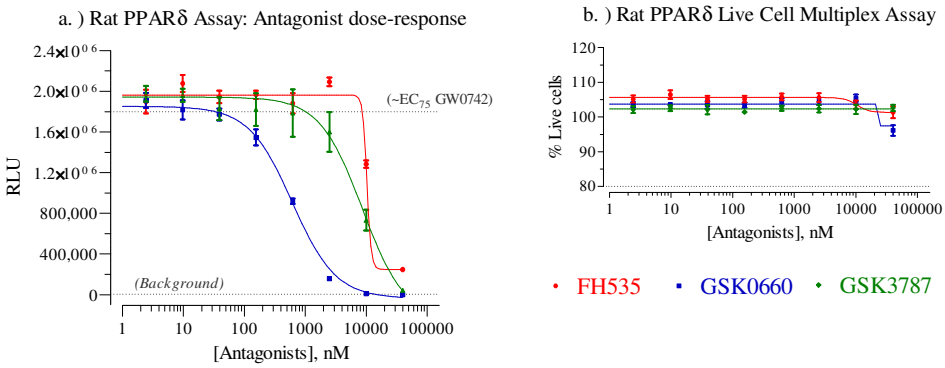


Figure 3. Validation of rPPARδ *Antagonist* dose-responses performed in combination with INDIGO's Live Cell Multiplex Assay.

(a.) Rat PPARδ antagonist assays performed using reference antagonists GSK0660, FH535, and GSK3787 (all from Tocris). **(b.)** To confirm that the observed drop in RLU values resulted from receptor inhibition, 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).

In brief: CSM was first used to prepare serial 4-fold dilutions of each antagonist to generate the desired range of 2x-concentration treatment media. Next, frozen Rat PPARδ Reporter Cells were thawed in CRM, supplemented with a '2x EC_{75} concentration' of GW0742, and 100 µl of this treated cell suspension was dispensed into each well of the assay plate. 100 µl of the prepared series of 2x-concentration treatment media were then dispensed per well, combining 1:1 with the reporter cells. Final assay concentrations of the respective antagonists ranged between 40 µM and 2.44 nM, including a 'no antagonist' control ($n \geq 6$ per treatment; highest [DMSO] $\leq 0.12\%$ *f.c.*). Each assay treatment contained ~ 156 nM (approximating EC_{75}) GW0742 as challenge agonist. 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 rPPARδ activity for each treatment condition.

Results: GSK0660, FH535, and GSK3787 all caused dose-dependent reduction in RLU values. The LCM Assay reveals no decrease in the numbers of live cells per assay well. Hence, the observed reductions in RLU values can be attributed to dose-dependent inhibition of rPPARδ activity by the test compounds, and *not* due to induced cell death.

NOTE: RLU values will vary slightly between different production lots of reporter cells and can vary *significantly* between different makes and models of luminometers.

II. Product Components & Storage Conditions

This rat PPARδ Reporter 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.

Kit Components	***Amount***	***Storage Temp.***
▪ Rat PPARδ Reporter Cells	3 x 0.60 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ GW0742, 10 mM (in DMSO) (reference agonist for rPPARδ)	1 x 30 µL	-20°C
▪ Detection Substrate	3 x 2.0 mL	**-80°C**
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate frame	1	ambient
▪ Snap-in, 8-well strips (white, sterile, cell-culture ready)	12	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

▪ dry ice bucket (*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-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 cell culture assay plate 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 9-15* 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_{50} – EC_{85}) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This rat PPARδ Reporter Assay kit includes a 10 mM stock solution of **GW0742**, an agonist of PPARδ that may be used to setup antagonist-mode assays. ~160 nM GW0742 typically approximates EC_{75} in this reporter 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: Prepare Test Compound treatment media for *Agonist-* or *Antagonist-mode* screens.

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

Note that, in *Step 6*, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl of Reporter Cells. Hence, to achieve the desired *final* assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Plan dilution volumes carefully; this kit provides 35 ml of CSM.

Preparing the positive control: This PPARδ Assay kit includes a 10 mM stock solution of the reference agonist **GW0742**. The following 8-point treatment series, with concentrations presented in 3-fold decrements, provides a suitable dose-response: 12000, 3000, 750, 188, 46.9, 11.7, and 2.93 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

Note that, in *Step 6*, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl of Reporter Cells. Hence, to achieve the desired *final* assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Use **CSM** to prepare the appropriate dilution series. Plan dilution schemes carefully. This assay kit provides 35 ml of CSM.

3.) 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 immerse in dry ice to transport the tube to a laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, and 3 tubes for 96 assay wells. W*ithout 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 and sanitize the outside surface of the tube with a 70% alcohol swab.

5.) ***a. Agonist*-mode assays**. Gently invert the tube of cells several times to disperse cell aggregates and gain a homogenous cell suspension. Without delay, dispense 100 µl / well of cell suspension into the strip-wells mounted in the plate frame.
~ 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 EC80-concentration of reference agonist (refer to "*A word about antagonist-mode assay setup*", pg. 8). Without delay, transfer the cell suspension into a reservoir and, using an 8-chanel pipette, 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 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 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 the appropriate number of vials of **Detection Substrate** *and* **Detection Buffer** from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.

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

9.) 30 minutes before intending to quantify rat PPARδ 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

Product No.	*Product Descriptions*
Cynomolgus Monkey PPARδ Assay Products	
C00121-32	Monkey PPARδ Reporter Assay 3x 32 assays in 8-well strips (96-well plate format)
C00121	Monkey PPARδ Reporter Assay 1x 96-well format assay
Dog PPARδ Assay Products	
D00121-32	Dog PPARδ Reporter Assay 3x 32 assays in 8-well strips (96-well plate format)
D00121	Dog PPARδ Reporter Assay 1x 96-well format assay
Rat PPARδ Assay Products	
R00121-32	Rat PPARδ Reporter Assay 3x 32 assays in 8-well strips (96-well plate format)
R00121	Rat PPARδ Reporter Assay 1x 96-well format assay
Mouse PPARδ Assay Products	
M00121-32	Mouse PPARδ Reporter Assay 3x 32 assays in 8-well strips (96-well plate format)
M00121	Mouse PPARδ Reporter Assay 1x 96-well format assay
Human PPARδ Assay Products	
IB00121-32	Human PPARδ Reporter Assay 3x 32 assays in 8-well strips (96-well plate format)
IB00121	Human PPARδ Reporter Assay 1x 96-well format assay
IB00122	Human PPARδ Reporter Assay 1x 384-well format assays
Panel of Human PPAR Assays	
IB00131-32P	PANEL of Human PPARγ, PPARα and PPARδ Assays 32 assays each in 8-well strips (96-well plate format)

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

VI. Limited Use Disclosures

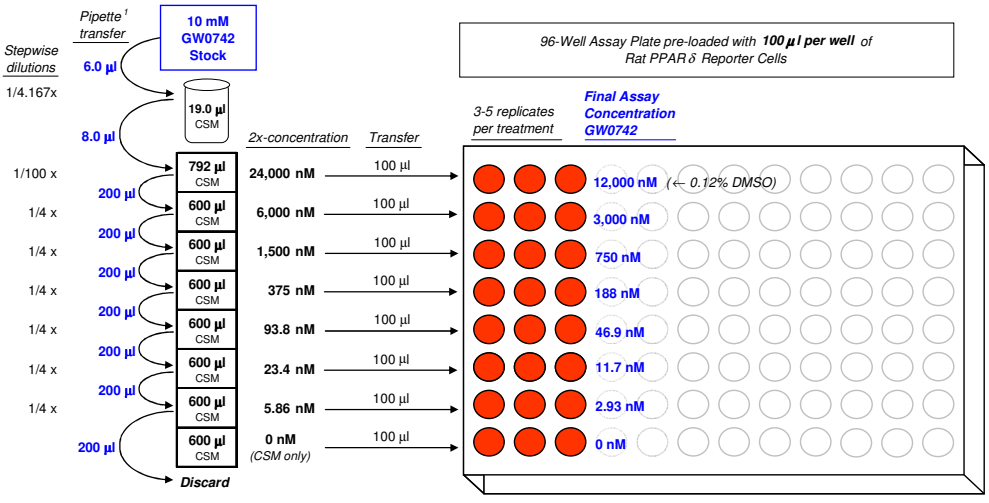
Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans.

"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.

APPENDIX 1

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



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