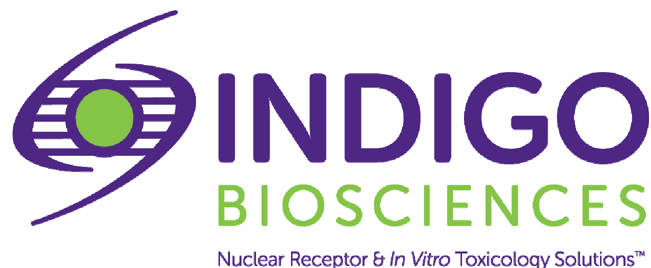


Mouse Peroxisome Proliferator-Activated Receptor Delta

(nr1c2, pparD, pparδ, pparβ)

Reporter Assay System

96-well Format Assays

Product # M00121

▪

Technical Manual

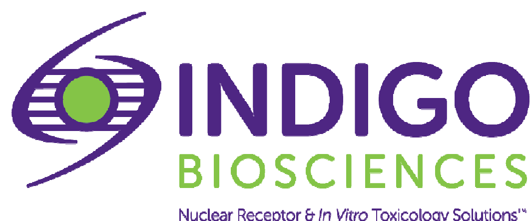
(version 7.1b)

www.indigobiosciences.com

3006 Research Drive, Suite A1, State College, PA 16801, USA

Customer Service:
814-234-1919; FAX 814-272-0152
customerserv@indigobiosciences.com

Technical Service:
814-234-1919
techserv@indigobiosciences.com



Mouse pparδ Reporter Assay System

96-well Format Assays

I. Description

▪ The Assay System……………………….……………….…….…...……..….3

▪ The Assay Chemistry……………………….……………….……..……......3

▪ Preparation of Test Compounds………….………………..………..………4

▪ Considerations for Automated Dispensing.…………….………..……..…4

▪ Assay Scheme...............................…………….............……….……....4

▪ Assay Performance……………………….……………….………..……..…5

II. Product Components & Storage Conditions ……………………….7

III. Materials to be Supplied by the User……………………...…...…7

IV. Assay Protocol

▪ A word about *Antagonist*-mode assay setup…………...…...............…...…8

▪ *DAY 1 Assay Protocol*……………………..…...…….……...…8

▪ *DAY 2 Assay Protocol*………………………...……….…......…9

V. Related Products…………………………………..……………..…...…..10

VI. Limited Use Disclosures……………………………………....……...11

APPENDIX 1: Example Scheme for Serial Dilution..………...…..……....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 **Mouse Peroxisome Proliferator-Activated Receptor Delta** (nr1c2), a ligand-dependent transcription factor commonly referred to as pparD or **pparδ**.

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

pparδ Reporter Cells are prepared using INDIGO's proprietary **CryoMite™** process. This cryo-preservation method yields exceptional cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for cumbersome intermediate treatment steps such as spin-and-rinse of cells, viability determinations, cell titer adjustments, or the pre-incubation of reporter cells prior to assay setup.
.
INDIGO Bioscience's Nuclear Receptor Reporter Assays are all-inclusive cell-based assay systems. In addition to pparδ Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, a reference agonist, Luciferase Detection Reagent, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

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

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (*Photinus pyralis*). Luciferase catalyzes the mono-oxidation of D-luciferin in a 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 Reporter Assay Systems feature a luciferase detection reagent specially formulated to provide stable light emission between 5 and 90+ minutes after initiating the luciferase reaction. Incorporating a 5 minute reaction-rest period ensures that light emission profiles attain maximal stability, thereby allowing assay plates to be processed in batch. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.

▪ Preparation of Test Compounds ▪

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using **Compound Screening Medium (CSM)**, as described in *Step 2* of the **Assay Protocol**. This method avoids the adverse effects of introducing high concentrations of DMSO into the assay. The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

NOTE: CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup, and are considered to be 'single-use' reagents.

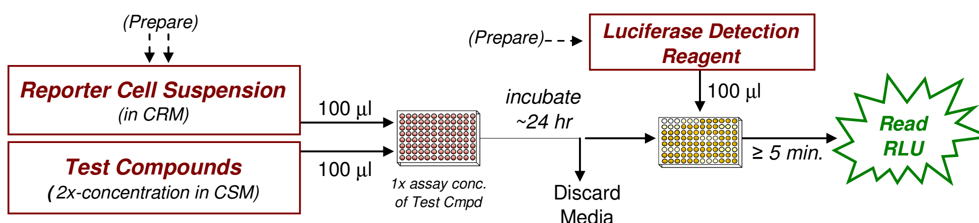
▪ Considerations for Automated Dispensing ▪

When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

Stock Reagent & Volume provided	Volume to be Dispensed *(96-well plate)*	Excess rgt. volume available for instrument dead volume
Reporter Cell Suspension 12 ml *(prepared from kit components)*	100 µl / well 9.6 ml / plate	~ 2.4 ml
LDR 12 ml *(prepared from kit components)*	100 µl / well 9.6 ml / plate	~ 2.4 ml

▪ 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.



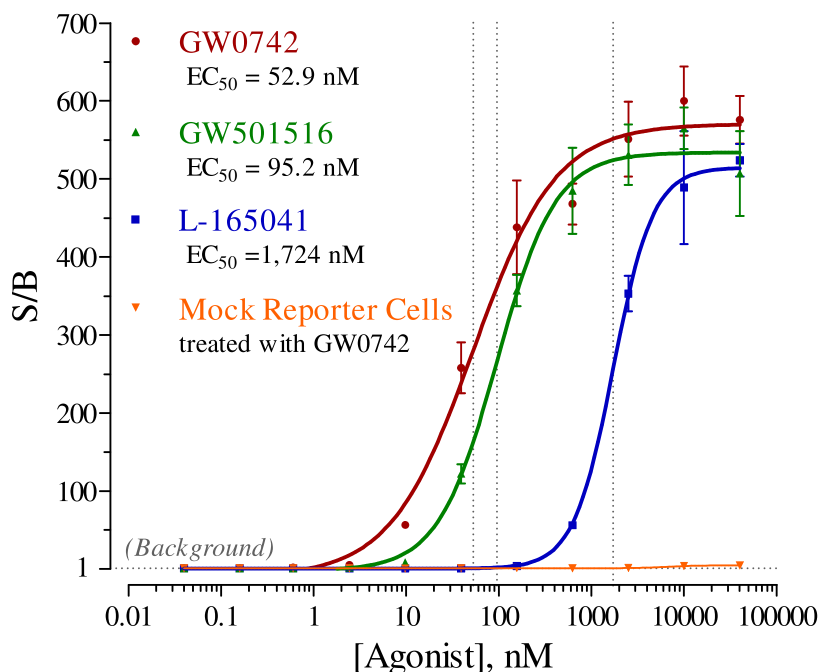


Figure 2. ***Agonist*** **dose-response of the mouse pparδ Assay.** pparδ assays were performed as described in this Technical Manual using manual dispensing of reference agonists GW0742 (provided), GW501516 and L-165041 (Tocris). In addition, to assess the level of background signal contributed by non-specific factors that may cause activation of the luciferase reporter gene, "Mock" reporter cells were specially prepared to contain only the luciferase reporter vector (mock reporter cells are not provided with assay kits). Concentrated stocks of agonists (in DMSO) were diluted in serial 4-fold decrements using CSM to generate 2x-concentration treatment media. Final assay concentrations of agonist treatment media ranged between 40 µM and 40 pM, and included a 'no-treatment' control (n ≥ 6 / treatment; highest [DMSO] ≤ 0.1% *f.c.* APPENDIX 1 describes an abbreviated 8-point dilution scheme that we find suitable for GW0742.) Mock Reporter Cells were identically treated with GW0742. Luminescence was quantified using a GloMax-Multi+ plate-reading luminometer (Promega Corp.). Average Relative Light Units (RLU) and their respective values of Standard Deviation (SD), Coefficient of Variation (CV), and Signal-to-Background (S/B) were determined for each treatment concentration. Z' values were calculated as described by Zhang, *et al.* (1999)[1]. Non-linear regression analyses were performed and EC_{50} values determined using GraphPad Prism software.

RESULTS: pparδ reporter cells treated with 10 µM GW0742 yielded an average RLU value with CV=7.4%, S/B ~ 590, and a corresponding Z'= 0.78. Mock reporter cells treated with GW0742 demonstrate no significant background luminescence (≤ 0.3% that of the reporter cells at EC_{Max}). Thus, luminescence results strictly through ligand-dependent activation of the pparδ expressed in these reporter cells.

[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.

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

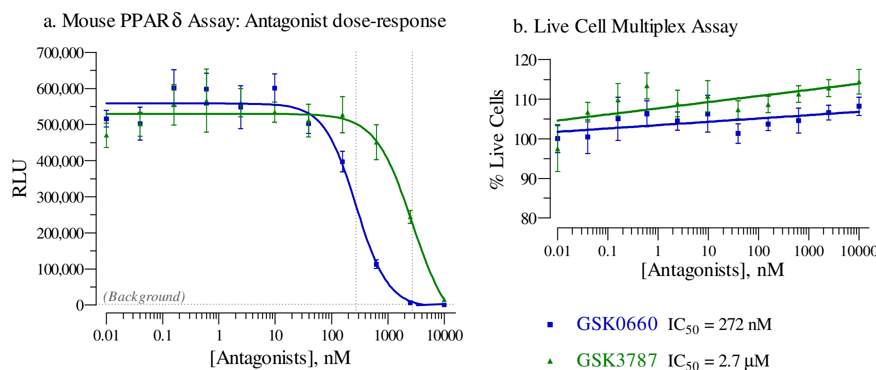


Figure 3. Mouse pparδ ***antagonist*** **dose-responses performed in combination with INDIGO's Live Cell Multiplex Assay. (a.)** pparδ antagonist assays were performed as described in this Technical Manual using manual dispensing of reference antagonists GSK0660 and GSK37887 (Tocris). **(b.)** To confirm 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 then used to prepare an 11-point, serial 4-fold dilution series of each antagonist to generate the desired range of 2x-concentration treatment media. Frozen pparδ Reporter Cells were then thawed in CRM and supplemented with a '2x-EC_{75}' concentration of GW0742. 100 µl of the reporter cell suspension was dispensed into each well of the assay plate. Next, 100 µl of the prepared treatment media series were dispensed per well, combining with the reporter cells. Final assay concentration of the challenge agonist approximated EC_{75}, while 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.*). Assay plates were incubated for ~23 hrs, then processed according to the LCM Assay protocol to quantify relative numbers of live cells per treatment condition. Plates were then further processed to quantify pparδ activity for each treatment condition.

Results: GSK0660 and GSK3787 both caused dose-dependent reduction in RLU values. The LCM Assay reveals no decrease in the numbers of live cells per assay well, up to the maximum treatment concentration of 10 µM. Hence, the observed reductions in RLU values can be attributed to dose-dependent inhibition of pparδ 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 mouse pparδ Reporter Assay System contains materials to perform assays in a single 96-well assay plate.

The aliquot of pparδ Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

Assay kits are shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels. Alternatively, the entire kit may be further stored at -80°C.

To ensure maximal viability, "Reporter Cells" must be maintained at -80°C until immediately prior to use.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit.

Kit Components	*Amount*	*Storage Temp.*
▪ mouse pparδ Reporter Cells	1 x 2.0 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 mouse pparδ)	1 x 30 µL	-20°C
▪ Detection Substrate	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-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_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).

▪ 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 $EC_{50} – EC_{85}$) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This pparδ Reporter Assay System kit includes a 10 mM stock solution of **GW0742**, an agonist of pparδ that may be used to setup antagonist-mode assays. 156 nM GW0742 typically approximates EC_{75} in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity. **APPENDIX 1** provides a guide for preparing CSM supplemented with the desired concentration of GW0742.

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 Reporter Assay System kit includes a 10 mM stock solution of the reference agonist **GW0742**. The following 8-point treatment series, with concentrations presented in 4-fold decrements, provides a suitable dose-response: 10000, 2500, 625, 156, 39.1, 9.77, 2.44 and 0.61 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 surface with a 70% ethanol swab.

Second, retrieve **Reporter Cells** from -80°C storage and, *without delay*, perform a rapid thaw of the frozen cells by transferring a 10 ml volume of the pre-warmed CRM into the tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 12 ml.

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

5.) ***a. Agonist*-mode assays**. Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, 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.

~ or ~

***b. Antagonist*-mode assays**. Gently invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain an homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "*A word about antagonist-mode assay setup*", pg. 8). 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 (100 µl/well) into a clear 96-well cell culture treated assay plate, followed by the addition of 100 µl/well of CSM (as in *Step 6*). 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 **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 mouse 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 an homogenous solution.

NOTE: Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.

10.) Set the plate-reader to "luminescence" mode. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, *or less*.

11.) *Immediately before proceeding to Step 12*, 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 following the addition of LDR. 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 performed in 5x 96-well plates
LCM-10	Reagent in 10x-bulk volume to perform **960** Live Cell Assays performed in 10x 96-well plates

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.

Copyright © INDIGO Biosciences, Inc. (State College, PA, USA). All Rights Reserved.

APPENDIX 1

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

10 mM GW0742 Stock

96-well Assay Plate pre-loaded with **100 µl per well** of Mouse PPAR δ Reporter Cells

Stepwise dilutions	*Pipette transfer*[1]	Volume	*'2x-concentration' treatment media*	*Transfer*	*3-5 replicates per treatment* — *Final Assay Concentration GW0742*
1/12.5 x	10.0 µl	115 µl CSM			
1/40 x	20.0 µl	760 µl CSM	20,000 nM	100 µl	10,000 nM (← 0.1% DMSO)
1/4 x	200 µl	600 µl CSM	5,000 nM	100 µl	2,500 nM
1/4 x	200 µl	600 µl CSM	1,250 nM	100 µl	625 nM
1/4 x	200 µl	600 µl CSM	313 nM	100 µl	156 nM
1/4 x	200 µl	600 µl CSM	78.1 nM	100 µl	39.1 nM
1/4 x	200 µl	600 µl CSM	19.5 nM	100 µl	9.77 nM
1/4 x	200 µl	600 µl CSM	4.88 nM	100 µl	2.44 nM
1/4 x	200 µl	600 µl CSM	1.22 nM	100 µl	0.610 nM
	200 µl	*Discard*			

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