Human Peroxisome Proliferator-Activated Receptor Gamma

(NR1C3, PPARG, PPARγ)

Reporter Assay System

96-well Format Assays
Product # IB00101

▪

Technical Manual
(version 7.1di)

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

Human PPARγ Reporter Assay System
96-well Format Assays

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 PPARG or **PPARγ**.

The N-terminal DNA binding domains (DBD) of the native PPARγ has been substituted with that of the yeast GAL4-DBD. The reporter gene is beetle luciferase functionally linked to the GAL4 upstream activation sequence (UAS). Thus, quantifying changes in luciferase expression in the treated reporter cells provides a specific and sensitive surrogate measure of the changes in PPARγ activity. The principal application of this reporter assay is in the screening of test samples to quantify any functional activity, either agonistic or antagonistic, that they may exert against 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 Reporter Assays are all-inclusive cell-based assays. 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, the reference agonist Rosiglitazone, Luciferase Detection Reagent, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's cell-based 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 assay kits 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 ▪

Small molecule test compounds are typically solvated in DMSO at high concentrations; ideally 1,000x-concentrated stocks relative to the highest desired treatment concentration in the assay. Using high-concentration stocks minimizes DMSO carry-over into the assay plates. Immediately prior to setting up an assay, the master stocks are serially diluted using one of two alternative strategies:

1.) As described in *Step 2* and depicted in Appendix 1 for the reference agonist Rosiglitazone, **Compound Screening Medium (CSM)** may be used as the diluent to make serial dilutions of test compounds to achieve the desired final assay concentration series.

Alternatively, if test compound solubility is expected to be problematic,
2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the final concentration of total DMSO carried over into assay wells should not exceed 0.4%. Significant DMSO-induced cytotoxicity can be expected above 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.

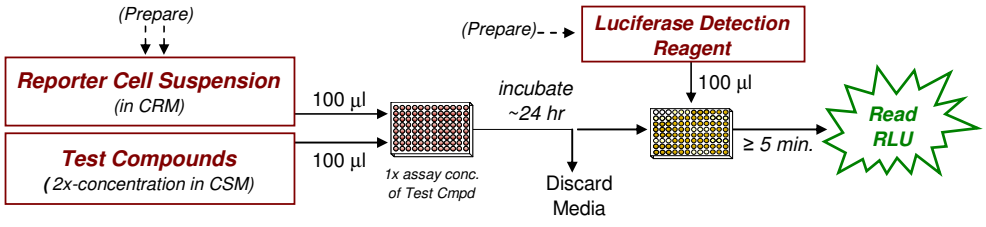
▪ Considerations for Automated Dispensing ▪

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.



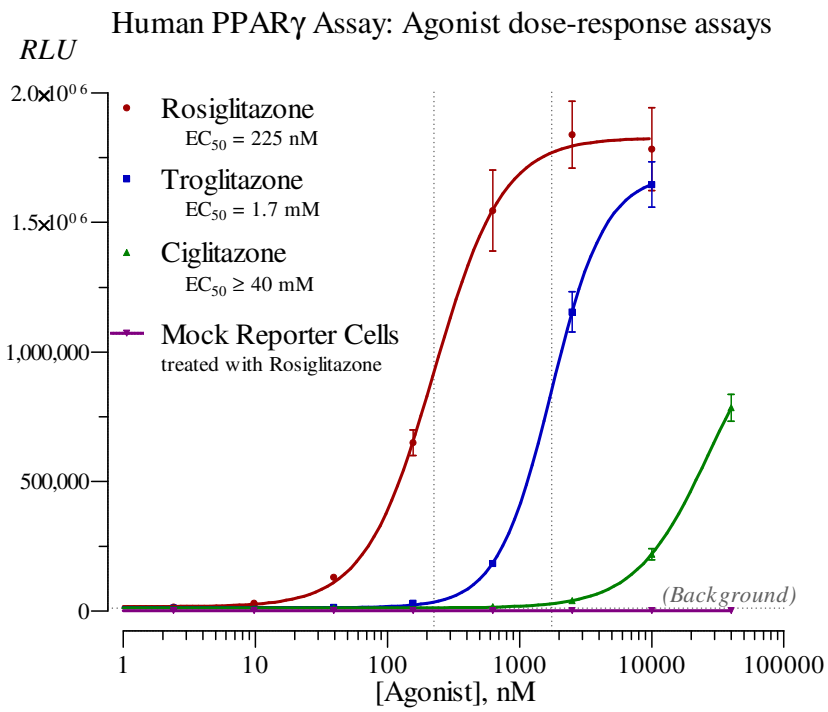


Figure 2a. Agonist dose-response analyses of the Human PPARγ Assay.
Validation of the PPARγ Assay was performed using manual dispensing and following the protocol described in this Technical Manual, using the reference agonists Rosiglitazone (provided), Troglitazone (Tocris) and Ciglitazone (Tocris). 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). 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)[1]. Non-linear regression analyses were performed and EC_{50} 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 EC_{Max}). Thus, luminescence results strictly through ligand-activation of the PPARγ 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.

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

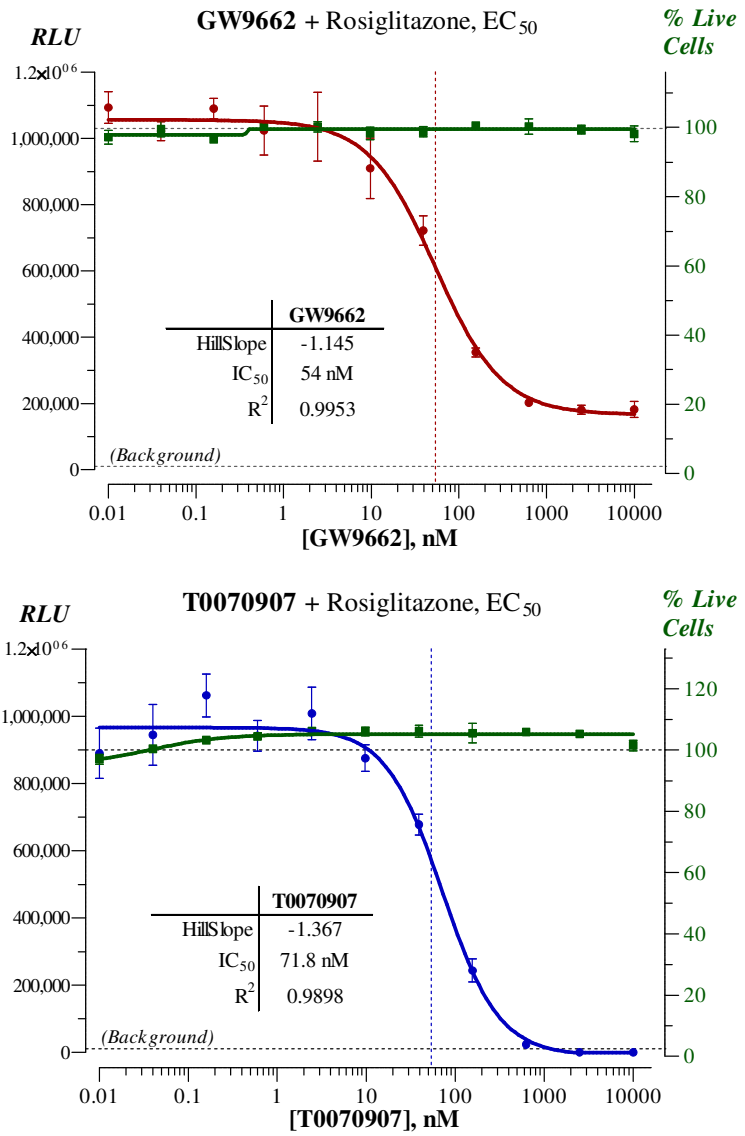


Figure 2b. ***Antagonist*** **dose-response analyses of Human PPARγ performed in combination with the INDIGO Live Cell Multiplex Assay.**
Antagonist assays were performed using T0070907 (Tocris), and GW9662 (Tocris). To confirm that the observed drop in RLU values resulted from receptor inhibition, as 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 ($n \geq 6$ per treatment; highest [DMSO] $\leq 0.15\%$ *f.c.*). Each treatment also contained 220 nM (approximating EC_{50}) 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 Reporter assay contains materials to perform assays in a single 96-well assay plate.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in *Step 3* of 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 inspect and inventory the individual kit components be sure to first transfer and submerge the tube of reporter cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, reporter 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.

Kit Component	*Amount*	*Storage Temp.*
▪ PPARγ 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
▪ Rosiglitazone, 10 mM (in DMSO) (reference agonist for PPARγ)	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

▪ 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-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* sterilized 96 deep-well blocks (*e.g.,* Axygen Scientific, #P-2ML-SQ-C-S), *or* appropriate similar vessel for generating dilution series of reference 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 5.4*): Prepare Test Compound treatment media for *Agonist-* or *Antagonist-mode* screens.

Total DMSO carried over into assay reactions should not 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 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 gain a homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of Rosiglitazone (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: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'cell blank' wells (meaning cell-free, but containing 'Compound Screening Media') must be included in the assay plate to allow quantification of plate-specific fluorescence background (refer to the LCMA Technical Manual).

NOTE 5.2: 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 5.3: 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 5.4: For logistical reasons, 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*.

NOTE 5.5: If well-to-well variation due to 'edge-effects' is a concern this problem *may* be mitigated by dispensing sterile liquid into the *inter-well* spaces of the assay plate. Simply remove 1 tip from the 8-chanel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

6.) Dispense 100 µl of 2x-concentration treatment media into appropriate assay wells.

7.) Transfer the assay plate into a cell culture incubator (37°C, humidified 5% CO_2) for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 70%) 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	
Product No.	***Product Descriptions***
IB00101-32	Human PPARγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB00101	Human PPARγ Reporter Assay System 1x 96-well format assay
IB00102	Human PPARγ Reporter Assay System 1x 384-well format assays
Panel of Human PPAR Assays	
IB00131-32P	PANEL_Human PPARγ, PPARα and PPARδ Reporter Assay 32 assays each in 8-well strips (96-well plate format)
Mouse/Rat PPARγ Assay Products	
MR00101-32	Mouse/Rat PPARγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
MR00101	Mouse/Rat PPARγ Reporter Assay System 1x 96-well format assay
MR00102	Mouse/Rat PPARγ Reporter Assay System 1x 384-well format assays
Cynomolgus Monkey PPARγ Assay Products	
C00101-32	Cynomolgus Monkey PPARγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
C00101	Cynomolgus Monkey PPARγ Reporter Assay System 1x 96-well format assay
Zebrafish PPARγ Assay Products	
Z00101-32	Zebrafish PPARγ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
Z00101	Zebrafish Monkey PPARγ Reporter Assay System 1x 96-well format assay
Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay	
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 5 x 96-well assay plates
LCM-10	Reagent in **10x bulk volume** to perform **960** Live Cell Assays performed in 10 x 96-well assay plates
INDIGlo Luciferase Detection Reagent	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents in 10 mL, 25 mL, 50 mL, and 500 mL volumes

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, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most currently updated version.

APPENDIX 1

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