

**Mouse/Rat Peroxisome Proliferator-Activated
Receptor Gamma
(nr1c3, pparG, mrPPAR γ)**

Reporter Assay System

3x 32 Assays in 96-well Format
Product # MR00101-32

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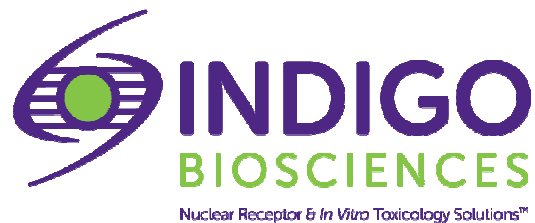
Technical Manual
(version 7.1bi)

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Mouse/Rat PPAR γ Reporter Assay System 3x 32 Assays in 96-well Format

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I. Description

▪ The Assay System ▪

This nuclear receptor assay utilizes a proprietary rodent cell line that is further engineered to express the **Mouse / Rat Peroxisome Proliferator-Activated Receptor Gamma** (nr1c3, pparG). Because the receptor's ligand binding domain sequence is conserved between mouse and rat species, the receptor is denoted herein as **mrPPAR γ** .

These mrPPAR γ Reporter Cells incorporate a responsive luciferase reporter gene, therefore, quantifying expressed luciferase activity provides a sensitive surrogate measure of mrPPAR γ activity in the treated cells. 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 mrPPAR γ .

mrPPAR γ Reporter Cells are prepared using INDIGO's proprietary **CryoMite™** process. This cryo-preservation method yields high cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for intermediate spin-and-wash steps, viability determinations, or cell titer adjustments.

INDIGO Bioscience's Nuclear Receptor Reporter Assays are all-inclusive cell-based assay kits. In addition to mrPPAR γ Reporter Cells, this kit provides two optimized media for use in thawing the frozen cells 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 O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, CO₂, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer, and is reported in terms of Relative Light Units (RLU's).

INDIGO's Nuclear Receptor Reporter 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 ▪

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 Dexamethasone, **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.

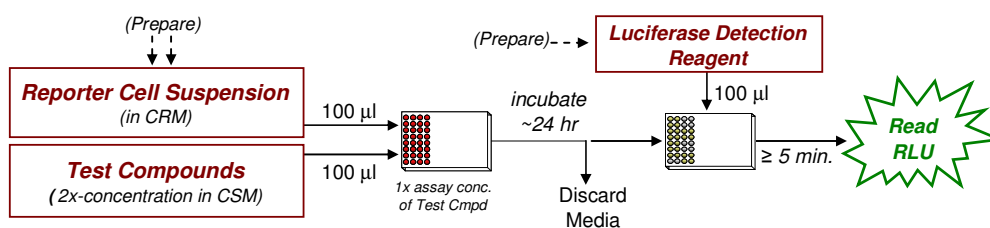
Alternatively, if test compound solubility is expected to be problematic, 2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared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

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, 100 μ l/well of Reporter Cells are dispensed into wells of the assay plate, followed immediately by dispensing 100 μ l/well of the prepared treatment media. Following 22-24 hours incubation, treatment media are discarded, and prepared Luciferase Detection Reagent (LDR) is added. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



▪ Assay Performance ▪

Mouse/Rat PPAR γ Assay: Agonist dose-response analyses

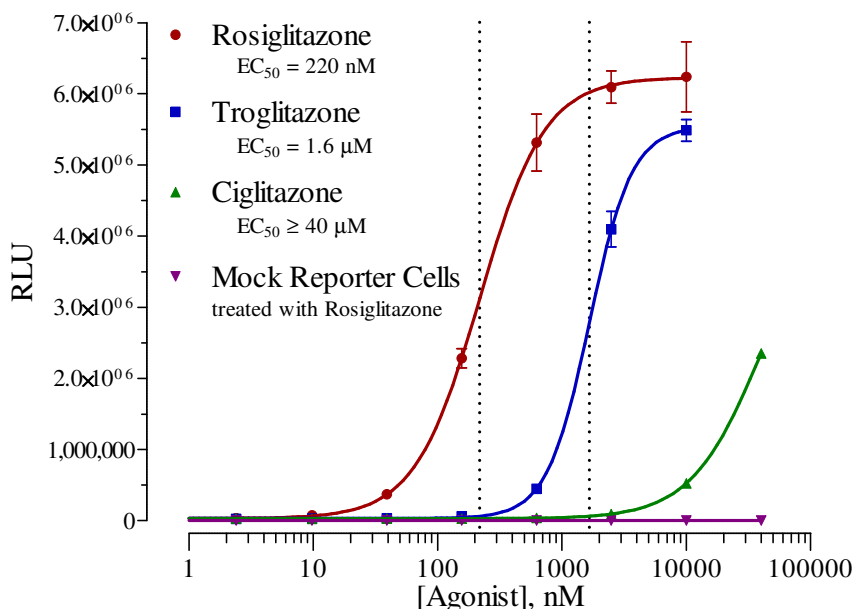


Figure 2a. Agonist dose-response of the Mouse/Rat PPAR γ Assay.

Validation of the mrPPAR γ Assay was performed 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). mrPPAR γ 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)¹. Non-linear regression analyses were performed and EC_{50} values determined using GraphPad Prism software.

RESULTS: mrPPAR γ reporter cells treated with 10,000 nM Rosiglitazone yielded an average RLU value with CV=7.9%, S/B = 248 and a corresponding Z' = 0.76. Similarly treated mock reporter cells demonstrate no significant background luminescence ($\leq 0.03\%$ that of the reporter cells at EC_{Max}). Thus, luminescence results strictly through ligand-activation of the mrPPAR γ 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.

$$Z' = 1 - [3 * (SD^{Reference\ max.} + SD^{Vehicle}) / (RLU^{Reference\ max.} - RLU^{Vehicle})]$$

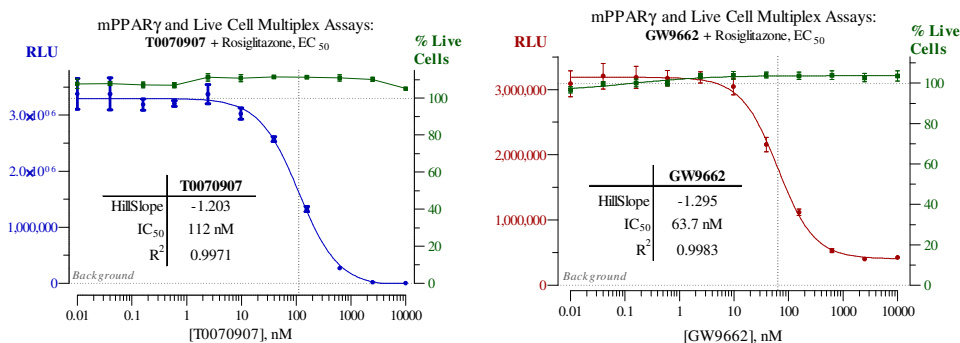


Figure 2b. Validation of Mouse/Rat PPAR γ antagonist dose-responses performed in combination with INDIGO's Live Cell Multiplex Assay.

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).

In brief: CSM supplemented with a '2x-EC₅₀ concentration' of Rosiglitazone was used to first prepare serial 4-fold dilutions of each antagonist to generate the desired range of 2x-concentration treatment media. mrPPAR γ Reporter Cells were then thawed in the presences of CRM and 100 μ l/well of cell suspension was dispensed into the assay plate. Next, 100 μ l of the prepared series of 2x-concentration treatment media were dispensed per well, thereby combining with the reporter cells. Final assay concentrations of the respective antagonists ranged between 10 μ 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 EC₅₀) Rosiglitazone as challenge agonist. Assay plates were incubated for 22-24 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 mrPPAR γ activity for each treatment condition.

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 μ M. Hence, the observed reduction in RLU values can be attributed to dose-dependent inhibition of mrPPAR γ 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 mouse/rat PPAR γ Reporter Assay kit contains materials to perform three distinct groups of assays in the format of a 96-well plate. 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.

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 2 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 please 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ mrPPAR γ 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
▪ Rosiglitazone, 10 mM (in DMSO) (reference agonist for PPAR γ)	1 x 30 μ L	-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% CO₂ 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 (refer to Fig 2b)
- *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 EC₅₀ – EC₈₅) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This mrPPAR γ assay kit includes a 10 mM stock solution of **Rosiglitazone**, an agonist of mrPPAR γ that may be used to setup antagonist-mode assays. 333 nM Rosiglitazone typically approximates EC₇₅ in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

Adding the reference agonist to the bulk suspension of Reporter Cells (*i.e.*, prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in *Step 5b* of the following protocol. Note that, in *Step 6*, 100 μ l of treatment media is combined with 100 μ 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 aseptic technique.

1.) Remove Cell Recovery Medium (CRM) and Compound Screening Medium (CSM) from freezer storage and thaw in a 37°C water bath.

2.) Prepare dilutions of treatment compounds (first see *Note 5.3*): Prepare Test Compound treatment media for *Agonist-* or *Antagonist-mode* screens.

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

Note that, in *Step 6*, 100 μ l of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 μ 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. Manage dilution volumes carefully. This assay kit provides 35 ml of CSM.

Preparing the positive control: This mrPPAR γ assay kit includes a 10 mM stock solution of **Rosiglitazone**, a reference agonist of mrPPAR γ . The following 7-point treatment series, with concentrations presented in 3-fold decrements, provides a complete dose-response: 3000, 1000, 333, 111, 37.0, 12.4, and 4.12 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 with a 70% ethanol swab.

Second, retrieve **Reporter Cells** from -80°C storage and place them directly into dry ice to transport them to the laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, and 3 tubes for 96 assay wells. When ready to begin, transfer the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring 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, during the 5 - 10 minutes incubation period, work in the cell culture hood to *carefully* mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

4.) Retrieve the tube of Reporter Cell Suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab, then transfer it into the cell culture hood.

5.) If more than one tube of Reporter cells was thawed, combine them and gently invert several times to gain a homogenous cell suspension.

a. Agonist-mode assays. Without delay, dispense 100 μ l of cell suspension into each well of the assay plate.

~ or ~

b. Antagonist-mode assays. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "A word about antagonist-mode assay setup", pg. 8). Dispense 100 μ l 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 'blank' wells (meaning cell-free but containing 'CSM') must be included in the assay plate to allow quantification of fluorescence background (refer to the LCMA Technical Manual).

NOTE 5.2: Users sometimes wish to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed (100 μ l/well) into a clear 96-well cell culture treated assay plate, followed by 100 μ l/well of CSM. Incubated overnight in identical manner to those reporter cells contained in the white assay plate.

NOTE 5.3: Some users find it more convenient to plate the reporter cells first, 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 5.4: 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.

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

7.) Transfer the assay plate into a cell culture incubator (37°C, humidified, 5% CO₂) for 22 - 24 hours.

NOTE: Ensure a high-humidity ($\geq 70\%$) environment within the 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 aseptic technique and may be performed on a bench top.

9.) 30 minutes before intending to quantify mrPPAR γ activity, remove **Detection Substrate** from the refrigerator and place them in a low-light area so that it may equilibrate to room temperature.

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 μ l 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

<i>Product No.</i>	<i>Product Descriptions</i>
Human PPARγ Assay Products	
IB00101-32	Human PPAR γ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB00101	Human PPAR γ Reporter Assay System 1x 96-well format assay
IB00102	Human PPAR γ Reporter Assay System 1x 384-well format assays
Panel of Human PPAR Assays	
IB00131-32P	PANEL_Human PPAR γ , PPAR α and PPAR δ Reporter Assay 32 assays each in 8-well strips (96-well plate format)
Mouse/Rat PPARγ Assay Products	
MR00101-32	Mouse/Rat PPAR γ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
MR00101	Mouse/Rat PPAR γ Reporter Assay System 1x 96-well format assay
MR00102	Mouse/Rat PPAR γ Reporter Assay System 1x 384-well format assays
Cynomolgus Monkey PPARγ Assay Products	
C00101-32	Cynomolgus Monkey PPAR γ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
C00101	Cynomolgus Monkey PPAR γ Reporter Assay System 1x 96-well format assay
Zebrafish PPARγ Assay Products	
Z00101-32	Zebrafish PPAR γ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
Z00101	Zebrafish Monkey PPAR γ 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.

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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.

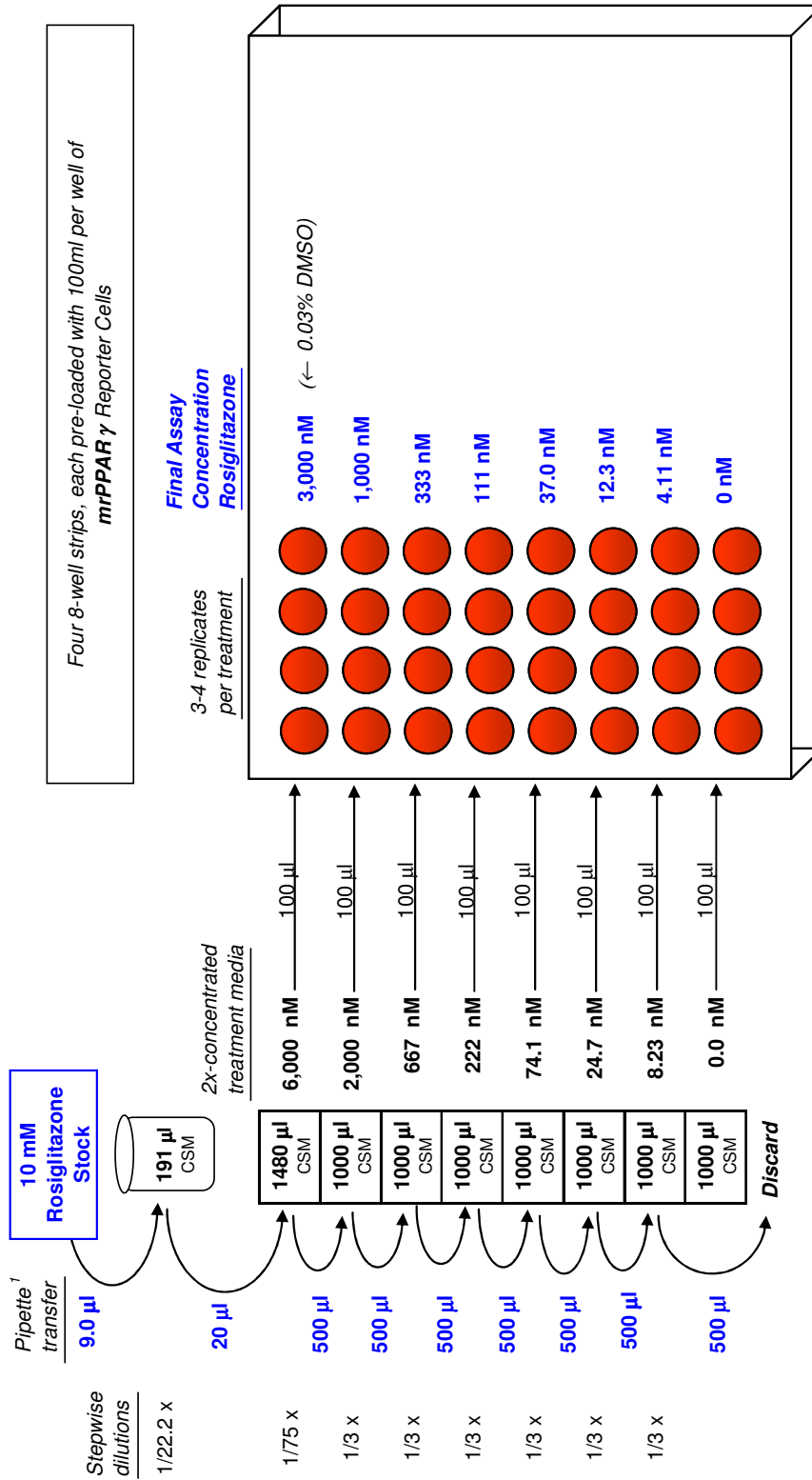
“CryoMite” is a Trademark TM of INDIGO Biosciences, Inc. (State College, PA, USA).

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.

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APPENDIX 1

Example scheme for the serial dilution of Rosiglitazone reference agonist, and the setup of a mrPPAR γ 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.