Mouse/Rat Peroxisome Proliferator-Activated Receptor Gamma

(nr1c3, pparG, mrPPARγ)

Reporter Assay System

96-well Format Assays
Product # MR00101

▪

Technical Manual
(version 7.1)

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Mouse/Rat PPARγ Reporter Assay System
96-well Format Assays

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I. Description

▪ The Assay System ▪

This nuclear receptor assay system 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 **mrPPARγ**.

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

mrPPARγ 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 systems. In addition to mrPPARγ 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 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.

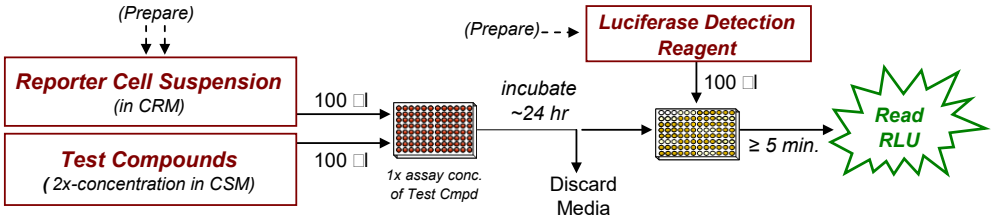
▪ Considerations for Automated Dispensing ▪

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.



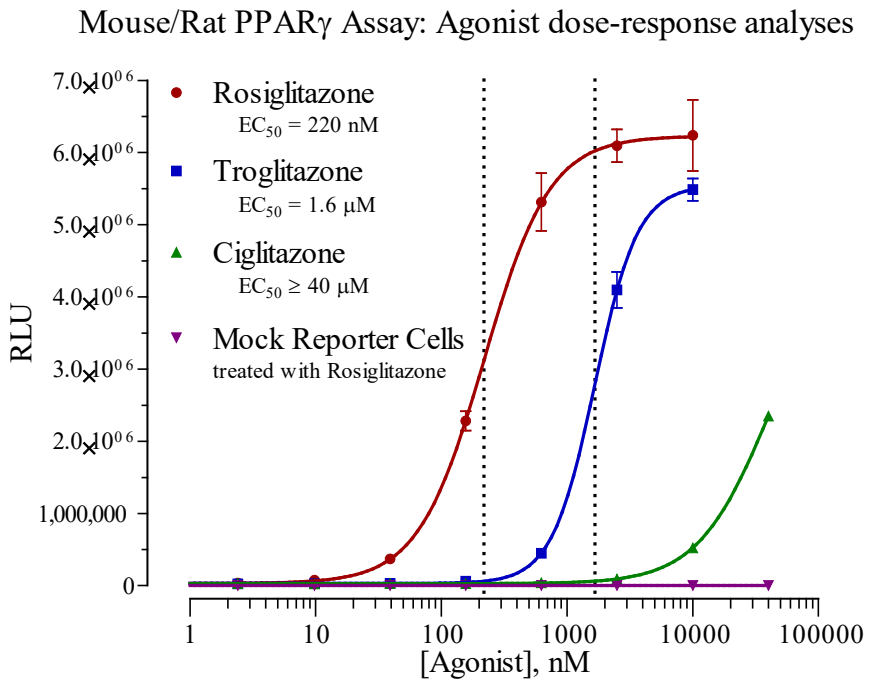


Figure 2a. Agonist dose-response of the Mouse/Rat PPARγ Assay.

Validation of the mrPPARγ 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). mrPPARγ 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: mrPPARγ 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 (≤ 0.03% that of the reporter cells at EC_{Max}). Thus, luminescence results strictly through ligand-activation of the mrPPARγ 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.

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

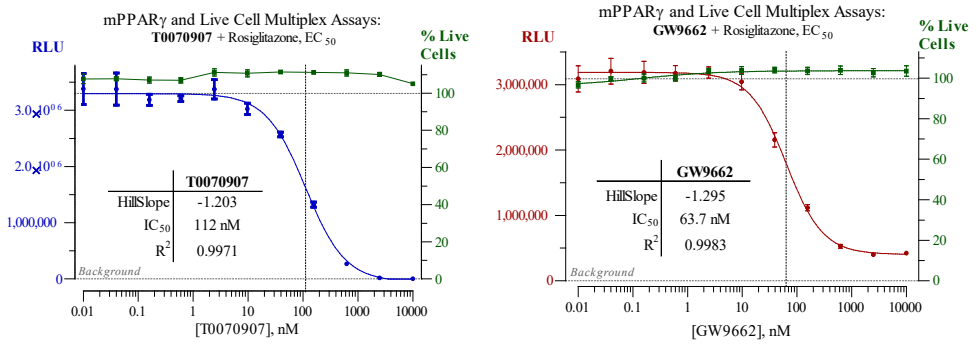


Figure 2b. Validation of Mouse/Rat PPARγ *antagonist* dose-responses performed in combination with INDIGO's Live Cell Multiplex Assay.

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-EC_{50} 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. mrPPARγ Reporter Cells were then thawed in the presences of CRM and 100 µl/well of cell suspension was dispensed into the assay plate. Next, 100 µl 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 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 22-24 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 mrPPARγ 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 µM. Hence, the observed reduction in RLU values can be attributed to dose-dependent inhibition of mrPPARγ 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 PPARγ Reporter Assay System contains materials to perform assays in a single 96-well assay plate.

The aliquot of 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.***
▪ mrPPARγ 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

▪ 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 mrPPARγ Reporter Assay System kit includes a 10 mM stock solution of **Rosiglitazone**, an agonist of mrPPARγ that may be used to setup antagonist-mode assays. 333 nM Rosiglitazone 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.

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 proper aseptic technique

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

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

The final concentration of 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). 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 mrPPARγ Reporter Assay System kit includes a 10 mM stock solution of **Rosiglitazone**, a reference agonist of mrPPARγ. The following 7-point treatment series, with concentrations presented in 3-fold decrements, provides a suitable 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 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, dispense 100 µl of cell suspension into each well of the 96-well 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). Dispense 100 µl of cell suspension into each well of the 96-well 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 aseptic technique, and may be performed on a bench top.

9.) 30 minutes before intending to quantify mrPPARγ 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

Mouse/Rat PPARγ Assay Products	
Product No.	***Product Descriptions***
MR00101-32	Mouse/Rat PPARγ Reporter Assay System 3x 32 assays in 96-well 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

Panel of Mouse PPAR Assays	
Product No.	***Product Description***
MR00131-32P	mrPPARγ, mPPARα and mPPARδ Reporter Assay PANEL 32 assays each in 1x 96-well plate

Human PPARγ Assay Products	
Product No.	***Product Descriptions***
IB00101-32	Human PPARγ Reporter Assay System 3x 32 assays in 96-well format
IB00101	Human PPARγ Reporter Assay System 1x 96-well format assay
IB00102	Human PPARγ Reporter Assay System 1x 384-well format assays
Bulk Reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

Panel of Human PPAR Assays	
Product No.	***Product Description***
IB00131-32P	Human PPARγ, PPARα and PPARδ Reporter Assay PANEL 32 assays each in 1x 96-well plate

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.

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VI. Limited Use Disclosures

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

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Product prices, availability, specifications and claims are subject to change without prior notice.

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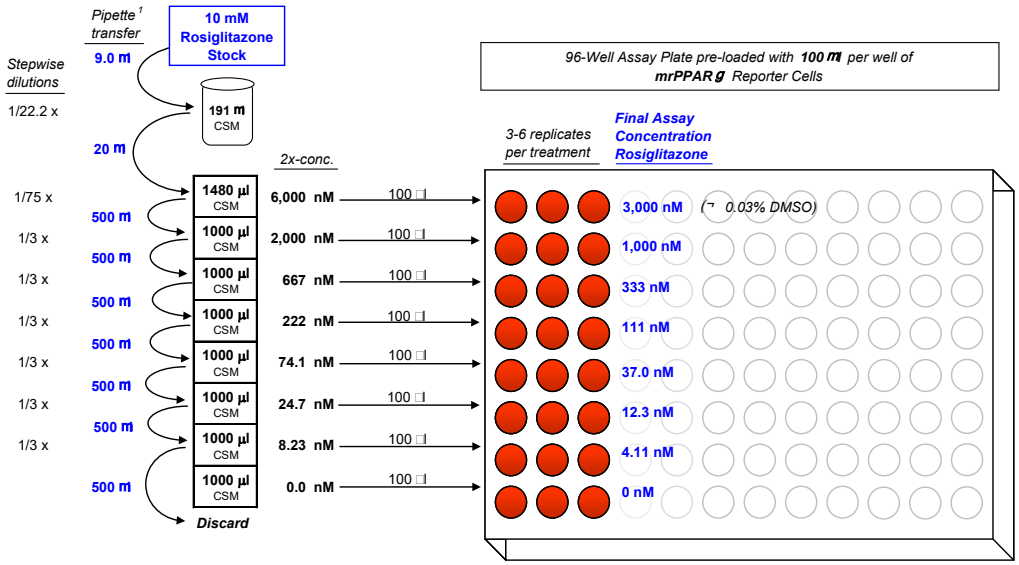
APPENDIX 1

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