

**Human Peroxisome Proliferator-Activated
Receptor Gamma
(NR1C3, PPARG, PPAR γ)**

Reporter Assay System

3x 32 Assays in 96-well Format
Product #IB00101-32

▪

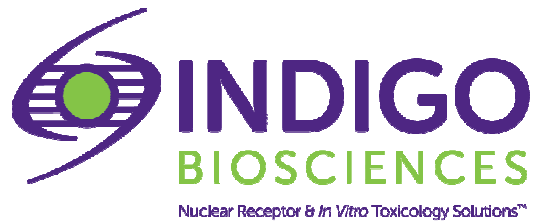
Technical Manual
(version 7.1m)

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

| | |
|--|----|
| I. Description | |
| ▪ The Assay System..... | 3 |
| ▪ The Assay Chemistry..... | 3 |
| ▪ Preparation of Test Compounds..... | 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 <i>Antagonist</i> -mode assay setup..... | 8 |
| ▪ <i>DAY 1 Assay Protocol</i> | 8 |
| ▪ <i>DAY 2 Assay Protocol</i> | 10 |
| V. Related Products | 11 |
| VI. Limited Use Disclosures | 12 |
| APPENDIX 1: Example Scheme for Serial Dilution | 13 |

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 PPAR γ or **PPAR γ** .

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to a PPAR γ -responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in PPAR γ activity. The principal application of this assay is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against human PPAR γ .

PPAR γ 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 assays are all-inclusive cell-based assay. In addition to PPAR γ 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 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 assay 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 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. The final concentration of DMSO carried over into assay wells should not exceed 0.4%.

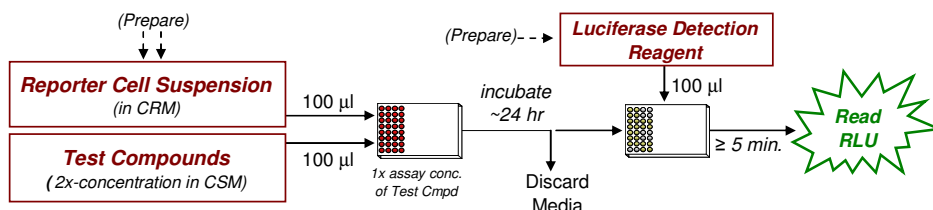
For protein or antibody samples it is recommended to solvate the materials in aqueous buffered solutions supplemented with carrier protein (*e.g.*, PBS + 0.1% BSA) at concentrations *no less* than 10x relative to the highest desired treatment concentration.

Immediately prior to setting up an assay the prepared stocks are serially diluted using **Compound Screening Medium (CSM)** to achieve the desired assay concentrations, as described in *Step 7*.

NOTE: CSM is formulated to help stabilize hydrophobic small molecule test compounds in the aqueous environment of the treatment media. Nonetheless, high concentrations of small organic molecules diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that compound dilutions are prepared in CSM immediately prior to assay setup and are then treated as '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 ▪

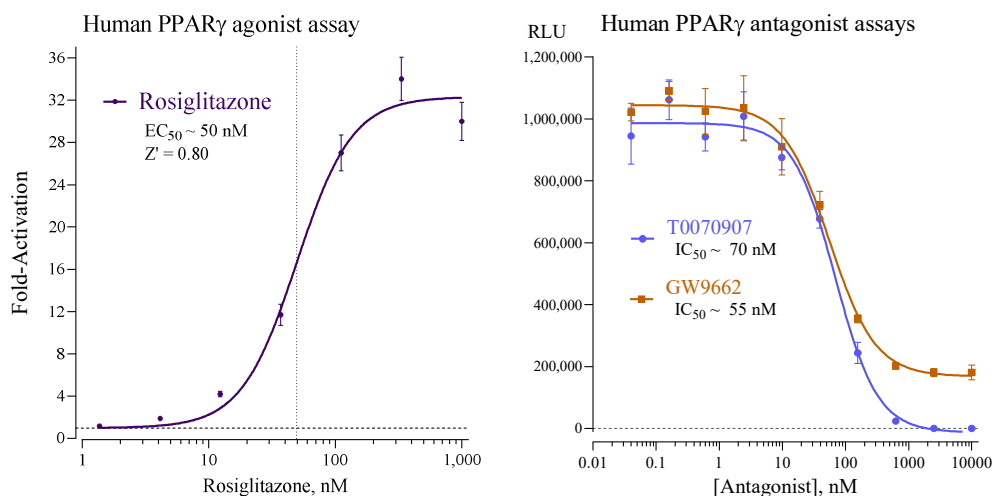


Figure 2. Agonist and Antagonist dose-response of Human PPAR γ . For the *agonist assay*, Reporter Cells were treated with the reference agonist Rosiglitazone (provided). For the *antagonist assays*, Reporter Cells were co-treated with a fixed EC80 concentration of the rosiglitazone and varying concentrations of the reference antagonists T007097 and GW9662. Average Relative Light Units (RLU), Average Fold-Activation, and respective values of Standard Deviation (SD) were calculated for each treatment concentration (n =4). Z' was calculated as per Zhang, *et al.* (1999)¹.

Treatment concentrations were Log10 transformed and GraphPad Prism was used to curve-fit data using the least-squares method of non-linear regression, and EC₅₀ and IC₅₀ values were determined.

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.

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

II. Product Components & Storage Conditions

This Human PPAR γ 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> |
|---|----------------|----------------------|
| ▪ PPAR 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, 1.0 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

- container of dry ice (used in 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.
- *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 varying concentrations of the test compound(s) to be evaluated for antagonist activity. This PPAR γ assay kit includes a 1.0 mM stock solution of **Rosiglitazone**, an agonist of PPAR γ that may be used to setup antagonist-mode assays. 120 nM Rosiglitazone typically approximates EC₈₀ in this assay. Hence, it presents a suitable 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 assay kit includes a 1.0 mM stock solution of **Rosiglitazone**, a commonly cited reference agonist of PPAR γ . The following 7-point treatment series, with concentrations presented in 3-fold decrements, provides a complete dose-response: 1000, 333, 111, 37.0, 12.3, 4.12, and 1.37 nM. Always include a 'no treatment' (or 'vehicle') control.

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 µl 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. 7). 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 '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 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 5.3: 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 µl 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 (≥ 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 or in a **fume hood**.

9.) 30 minutes before intending to quantify receptor 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. Program the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time is 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.

NOTE: 'Detection Substrate' contains a high concentration of DTT, which produces a strong odor that some users may find objectionable. It is advised to work in a **fume hood** when preparing LDR, and subsequently when dispensing it into the assay plate and throughout the following 'plate rest' period (*Step 13*).

12.) After 22-24 hours of incubation, remove media contents from each well.

NOTE: Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. Do *not* touch the well bottom or run the tip of the aspiration device around the bottom circumference of the assay well. Such practices will result in destruction of the reporter cells and greatly increased well-to-well variability. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using an 8-pin manifold (*e.g.*, Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus.

13.) Add 100 µl of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for 5 - 10 minutes. Do not shake the assay plate during this period.

14.) Quantify luminescence.

15.) Data analyses.

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, 500 mL, and larger custom 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 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 current version.

Copyright © INDIGO Biosciences, Inc. (State College, PA, USA). All rights reserved.

