Human Peroxisome Proliferator-Activated Receptor Assays

PANEL

PPARα, PPARδ, PPARγ

32 Assays each in 96-well Format
Product #IB00131-32P

▪

Technical Manual
(version 7.2m)

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Human PPAR Assays PANEL
PPARα, PPARδ, PPARγ
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I. Description

▪ The Assay System ▪

INDIGO's **PANEL** of **PPAR Reporter Assays** utilizes non-human mammalian cells engineered to express **Human Peroxisome Proliferator-Activated Receptors**: **PPARα** (NR1C1), **PPARδ** (NR1C2), or **PPARγ** (NR1C3), all ligand-dependent transcription factors that are commonly referred to as **PPARα**, **PPARδ** and **PPARγ**.

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

PPAR 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 or cell titer adjustments prior to assay setup.

INDIGO's nuclear receptor Assays are all-inclusive cell-based assay systems. In addition to PPAR 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 assay kits 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).

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 ▪

Test compounds are typically solvated at high concentrations in DMSO and stored frozen as master stocks. Immediately prior to setting up an assay, the master stocks are serially diluted using one of two alternative strategies:

1.) As described in *Step 8* and depicted in Appendix 1 for the reference agonists, **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.

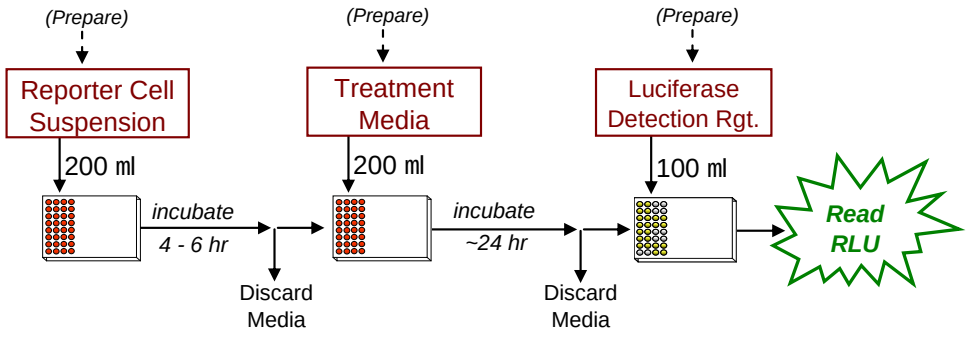
Regardless of the dilution method used, the final concentration of total DMSO, or any organic solvent, carried over into assay wells should not exceed 0.4%. Significant DMSO-induced cytotoxicity can be expected above 0.4%.

(continued)

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*, 200 µl of Reporter Cells is dispensed into wells of the assay plate, which is then pre-incubated for 4 - 6 hours. Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared 1x-concentration treatment media are added. Following 22 - 24 hours incubation, treatment media are discarded, and Luciferase Detection Reagent 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 ▪

Figures 2, **3**, and **4** present performance data for the PPARα, PPARδ, and PPARγ assays. To assess the level of background signal contributed by non-specific factors that may cause activation of the luciferase reporter gene, "mock" reporter cells, which contain only the luciferase vector, were treated with agonist, as noted in respective figures (mock reporter cells are not provided with assay kits). Luminescence was quantified ($n \geq 4$). Respective average RLU values were normalized to the untreated control RLU values. Data were plotted as Fold-Activation (error bars depict +/- %CV) *vs.* Log10 agonist (nM) using non-linear regression. Data plotting and EC_{50} determinations were performed using GraphPad Prism software. Z' values were calculated as described by Zhang, *et al*. (1999)[1].

[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.

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

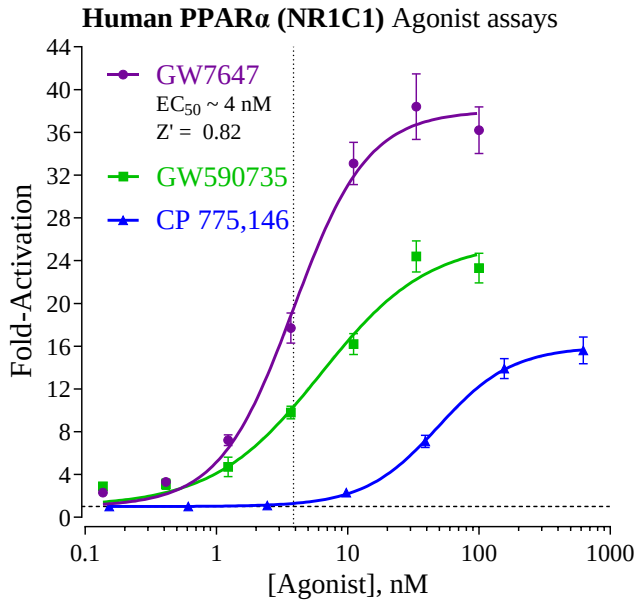


Figure 2. ***Agonist*** **dose-response analyses of Human PPARα.**
Analyses of PPARα Reporter Cells using the reference agonists GW7647 (provided), GW590735 and CP 775,146 (Tocris). **Appendix 1** describes an abbreviated 8-point dilution scheme for GW7647.

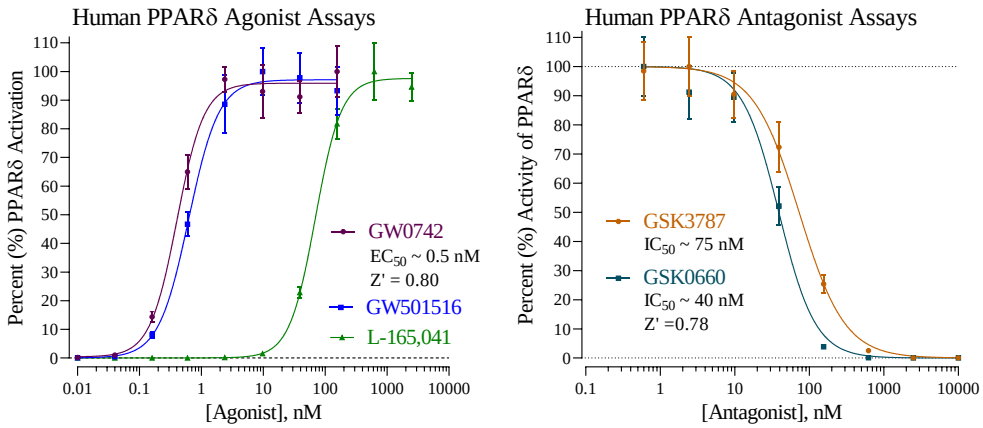


Figure 3. *Agonist* and *Antagonist* dose-response of the PPARδ receptor.
PPARδ reference *agonists* GW0742 (provided), GW501516 and L-165041 (Tocris), and *antagonists* GSK3787 and GSK0660 (Tocris) were analyzed.

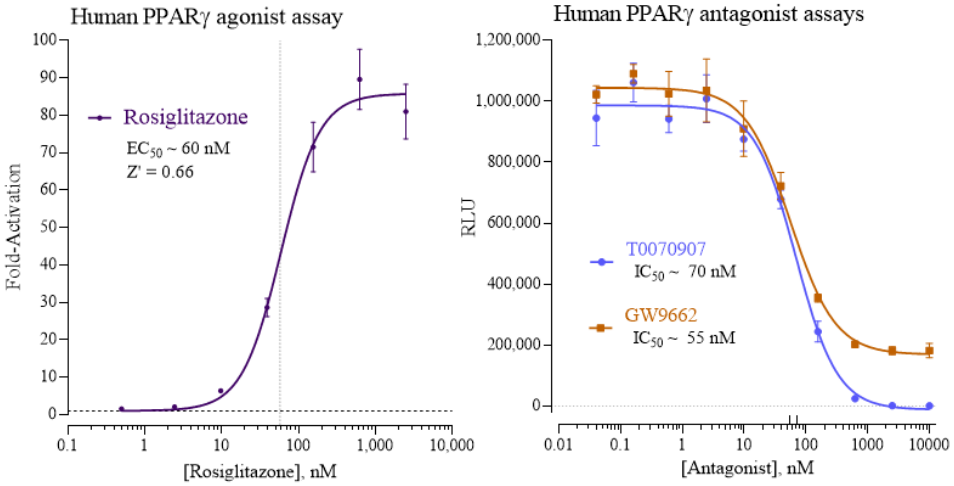


Figure 4. *Agonist* and *Antagonist* dose-response of Human PPARγ. Reporter Cells were treated with the reference agonist Rosiglitazone (provided) and the antagonists T007097 andGW9662. Values of average Relative Light Units (RLU) and respective Standard Deviation (SD) were calculated for each treatment concentration (n =4). Z' was calculated as per Zhang, *et al*. (1999)[1].

II. Product Components & Storage Conditions

This Human PPAR Assays PANEL contains materials to perform 32 PPARα assays, 32 PPARδ assays, and 32 PPARγ assays, all in a single 96-well plate format. All reagents are supplied with sufficient extra volume to accommodate the needs of performing 3 individual groups of 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 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.

Aliquots of Reporter Cells are provided as a single-use reagents. 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 Components	***Amount***	***Storage Temp.***
▪ PPARα Reporter Cells	1 x 0.60 mL	**-80°C**
▪ PPARδ Reporter Cells	1 x 0.60 mL	**-80°C**
▪ PPARγ Reporter Cells	1 x 0.60 mL	**-80°C**
▪ Cell Recovery Media (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Media (CSM)	1 x 45 mL	-20°C
▪ *PPARα reference agonist:* GW7647, 100 µM (in DMSO)	1 x 30 µL	-20°C
▪ *PPARδ reference agonist:* GW0742, 30 µM (in DMSO)	1 x 30 µL	-20°C
▪ *PPARγ reference agonist:* Rosiglitazone, 1.0 mM (in DMSO)	1 x 30 µL	-20°C
▪ Detection Substrate	3 x 2.0 mL	**-80°C**
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ 96-well format plate frame	1	ambient
▪ snap-in, 8-well strips (white, sterile, cell culture treated)	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 2*)
▪ 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:* reference antagonists
▪ *Optional:* clear 96-well assay plate, sterile, *collagen-coated*, 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 1-12* are performed on ***Day 1***, requiring less than 2 hours to complete. *Steps 13-18* 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 EC_{50} – EC_{85}) of a known *agonist* AND the test compound(s) to be evaluated for antagonist 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 the following protocol.

This PPAR Assay Panel kit provides a commonly used reference agonist for each PPAR assay; they may be used effectively to setup respective receptor inhibition assays.

▪ **PPARα**: GW7647 is provided as a 300 µM stock in DMSO; it may be used as an agonist of PPARα (Figure 2) to set up antagonist screens. 10 nM GW7647 typically approximates $EC_{60\text{-}70}$ in this cell-based reporter assay. Hence, it presents a suitable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

▪ **PPARγ**: GW0742 is provided as a 30 µM stock in DMSO; it may be used as an agonist of PPARδ (Figure 3) to set up antagonist screens. 3 nM GW0742 typically approximates $EC_{70\text{-}80}$ in this assay.

▪ **PPARγ**: Rosiglitazone is provided as a 1.0 mM stock in DMSO; it may be used as an agonist of PPARγ (Figure 4A) to set up antagonist screens. 155 nM Rosiglitazone typically approximates EC_{80} in this assay.

DAY 1 Assay Protocol: All steps must be performed using aseptic technique.

1.) Remove the **2 tubes** of **Cell Recovery Medium (CRM)** from freezer storage, thaw and equilibrate to 37°C using a water bath.

2.) Rapid Thaw of the Reporter Cells: *First*, retrieve the two tubes of **CRM** from the 37°C water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve **PPAR Reporter Cells** from -80°C storage and place them directly into dry ice to transport them to the laminar flow hood: each PPAR will utilize 32 assay wells. When ready, transfer the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring **6.4 ml** of pre-warmed 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. The resulting volume of respective PPAR cell suspensions will be **7.0 ml** per tube.

3.) 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 Cell Suspensions from the water bath and sanitize the outside surface with a 70% alcohol swab.

5.) Dispense **200 µl / well** of a specific PPAR cell suspension into 32-wells of the assay plate.

NOTE 5.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, '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: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the reservoir during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.

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 into a clear 96-well assay plate. Continue to process the assay plate in identical manner to the white assay plate.

6.) Pre-incubate reporter cells: Place the assay plate into a mammalian cell incubator (37°C, ≥70% humidity, 5% CO_2) for 4 - 6 hours.

7.) Near the end of the pre-incubation period remove **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.

8.) Prepare the Test Compound and Reference Compound treatment media at the desired final assay concentrations: Use **CSM** to prepare an appropriate dilution series of the reference and test compound stocks. Prepare all treatment media at the desired final assay concentrations. In *Step 10*, the prepared treatment media will be dispensed at 200 µl / well into the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO (or any other organic solvent) carried over into assay reactions should not exceed 0.4%.

***a. Agonist*-mode assays**. This PPAR Assay Panel kit provides a commonly used reference agonist for each PPAR assay.

▪ **PPARα.** Agonist GW7647 is provided as a 300 µM stock in DMSO. The following 7-point treatment series, with concentrations presented in 3-fold decrements, provides a suitable dose-response: 300, 100, 33.3, 11.1, 3.70, 1.23 and 0.412 nM (Figure 2). Always include a 'no treatment' (or 'Vehicle only') control. **APPENDIX 1** provides an example for generating such a dilution series.

▪ **PPARδ**. Agonist GW0742 is provided as a 30 µM stock in DMSO. The PPARδ assay typically exhibits a complete dose-response to GW0742 using treatment concentrations: 30, 10, 3.33, 1.11, 0.370, 0.123, and 0.0412 (Figure 3). Always include a 'no treatment' (or 'Vehicle only') control. **APPENDIX 2** provides an example for generating such a dilution series.

▪ **PPARγ**. Agonist Rosiglitazone is provided as a 1.0 mM stock in DMSO. The PPARγ assay typically exhibits a complete dose-response to Rosiglitazone using treatment concentrations: 1,000, 333, 111, 37.0, 12.3, 4.12, and 1.37 nM (Figure 4A). Always include a 'no treatment' (or 'Vehicle only') control. **APPENDIX 3** provides an example for generating such a dilution series.

~ or ~

***b. Antagonist*-mode assays**. When setting antagonist assays, first supplement a bulk volume of CSM with the appropriate challenge agonist to achieve the desired final assay-concentration (refer to "*A word about antagonist-mode assay setup*", pg. 9). The agonist-supplemented CSM is then used to generate dilutions of test compound samples to achieve their final assay concentrations.

9.) At the end of the cell pre-incubation period, discard the culture media. Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using either a single-tip, or an 8-pin manifold (*e.g.*, Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus. 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 cells and greatly increased well-to-well variability.

10.) Dispense **200 µl** of each prepared control media and treatment media into appropriate wells of the assay plate.

11.) Transfer the assay plate into a cell culture incubator 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.

DAY 2 Assay Protocol: Subsequent manipulations do *not* require special regard for aseptic technique and may be performed on an open bench top.

12.) 30 minutes before intending to quantify receptor activity, remove **Detection Substrate** and **Detection Buffer** from the freezer and place them in a low-light area so that they may thaw and equilibrate to room temperature.

NOTE: Do NOT actively warm Detection Substrate above room temperature. A room temperature water bath may be used to expedite thawing.

13.) 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*.

14.) *Immediately before proceeding to Step 15*: 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.

15.) Following 22 - 24 hours incubation in treatment media, remove media contents from each well of the assay plate (as before in *Step 9*).

16.) 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.

17.) Quantify luminescence.

V. Related Products

Product No.	*Product Descriptions*
Human PPARα Assay Products	
IB00111-32	Human PPARα Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB00111	Human PPARα Reporter Assay System 1x 96-well format assay
IB00112	Human PPARα Reporter Assay System 1x 384-well format assays
Human PPARδ Assay Products	
IB00121-32	Human PPARδ Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB00121	Human PPARδ Reporter Assay System 1x 96-well format assay
IB00122	Human PPARδ Reporter Assay System 1x 384-well format assays
Human PPARγ Assay Products	
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 assays
IB00102	Human PPARγ Reporter Assay System 1x 384-well format assays
Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

Panel of Human PPAR Assays	
IB00131-32P	Human PPARγ, PPARα and PPARδ Reporter Assay PANEL 32 assays each in 8-well strips (96-well plate format)

Product No.	*Product Descriptions*
Mouse/Rat PPARγ Assay Products	
MR00101-32	Mouse/Rat PPARα Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
MR00101	Mouse/Rat PPARα Reporter Assay System 1x 96-well format assay

Panel of Mouse PPAR Assay Products	
MR00131-32P	mrPPARγ, mPPARα and mPPARδ Reporter Assay PANEL 32 assays each in 1x 96-well plate

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

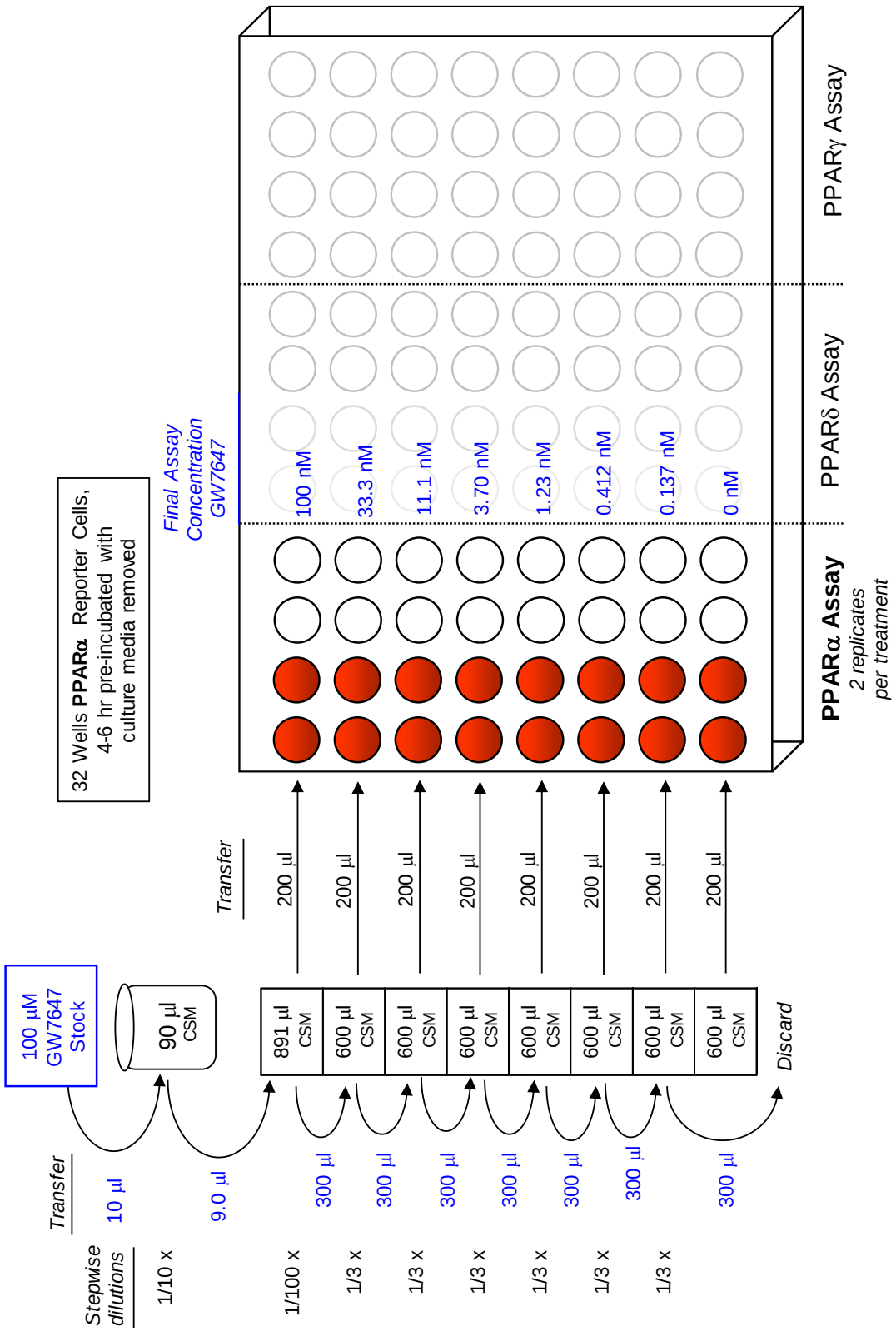
Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic, diagnostic, or contact use in humans or animals.

"CryoMite" is a Trademark ™ 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 recent version.

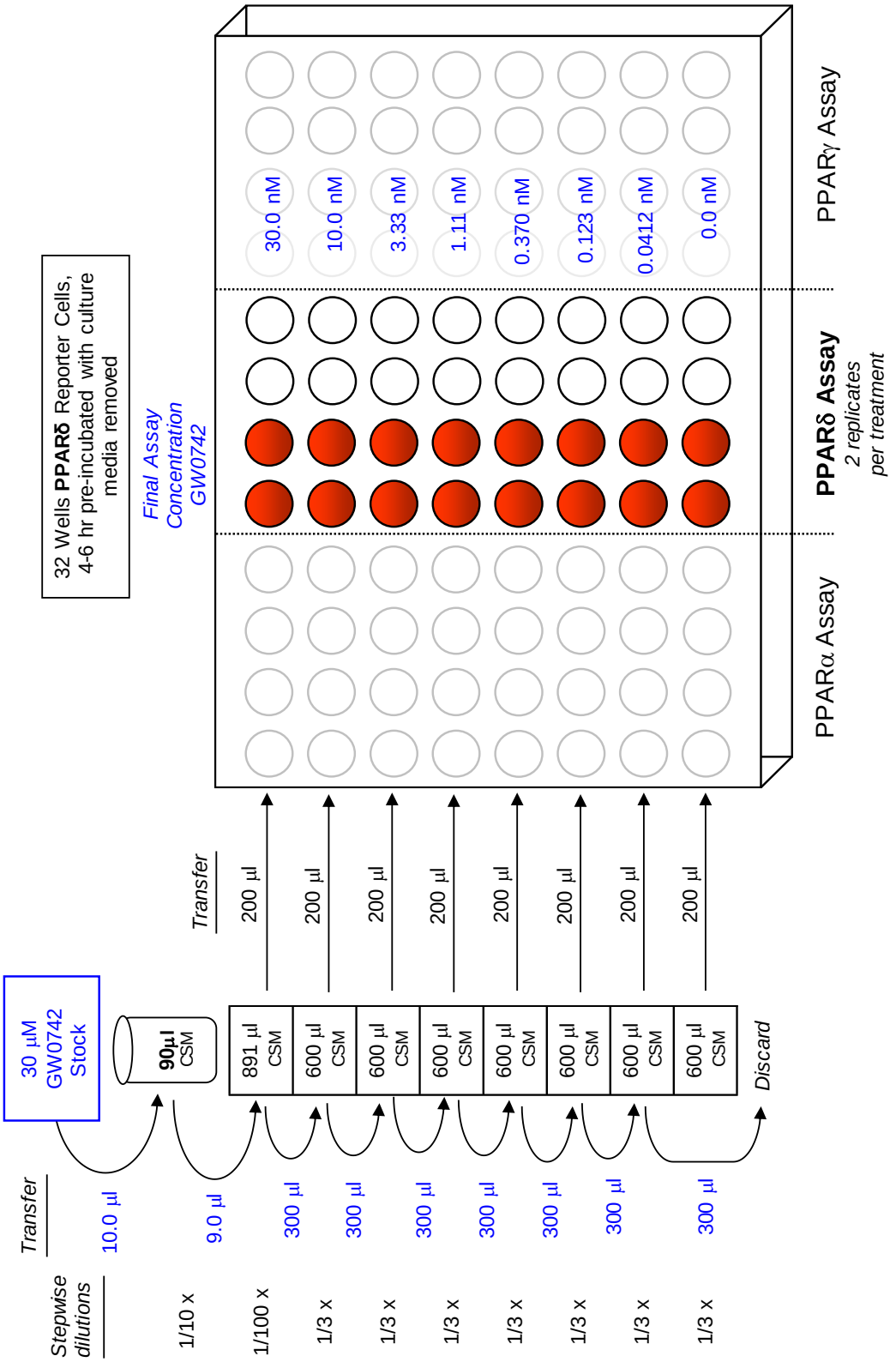
APPENDIX 1

Example scheme for the serial dilution of GW590735 reference agonist, and the setup of a PPARα dose-response assay.



APPENDIX 2

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



APPENDIX 3

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