



INDIGO Biosciences, Inc.

The Nuclear Receptor Company™

**Human Peroxisome Proliferator-Activated Receptor
Reporter Assays
PANEL**

PPAR α , PPAR δ , PPAR γ

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

■

Technical Manual

(version 3.0)

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Human PPAR Reporter Assays PANEL
PPAR α , PPAR δ , PPAR γ
32 Assays each in 96-well Format

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I. Description

INDIGO Bioscience's Nuclear Receptor Reporter Assays are all-inclusive cell-based assay systems. In addition to human nuclear receptor reporter cells, kits provide an optimized cell culture medium, a medium for diluting test compounds, a positive-control agonist specific to each nuclear receptor, luciferase detection reagent, and a cell culture-ready assay plate. The primary application of these reporter assays is in the screening of test compounds to quantify functional activities, either agonist or antagonist, that they may exert against a specific human nuclear receptor.

These assay systems utilize reporter cells 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.

This **PANEL of PPAR Reporter Assays** utilizes non-human mammalian cells engineered to express three distinct **Human Peroxisome Proliferator-Activated Receptors: PPAR α** (NR1C1), **PPAR δ/β** (NR1C2), and **PPAR γ** (NR1C3), as well as the luciferase reporter gene.

INDIGO's nuclear receptor assays capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology. These nuclear receptor 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 oxyluciferin, AMP, PP_i, CO₂, and photon emission. The luminescence intensity of the reaction is quantified using a plate-reading luminometer, and is reported in terms of relative light units (RLU).

In a typical assay setup, reporter cells are dispensed into wells of the assay plate and then immediately dosed with the user's test compounds. Following an overnight incubation, the treatment media are discarded and luciferase detection reagent (LDR) is added. The intensity of light emission from the ensuing luciferase reaction provides a sensitive measure that is directly proportional to the level of PPAR activation in the reporter cells.

All of INDIGO's Nuclear Receptor Reporter Assay Systems feature a luciferase detection reagent specially formulated to provide stable light emission between 15 and 90 minutes after initiating the luciferase reaction (refer to APPENDIX 2). There is no need to sequentially process-and-read single assay plates. The unwavering signal stability of these PPAR Nuclear Receptor Assays enables batch processing of large numbers of assay plates, making this reporter chemistry optimally suited to meet the logistical challenges inherent to high-throughput screening (HTS).

II. Product Components & Storage Conditions

This Human PPAR Reporter Assays PANEL contains materials to perform 32 PPAR α assays, 32 PPAR δ assays, and 32 PPAR γ 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.

The individual aliquots of PPAR Reporter Cells and Detection Solutions I & II are provided as single-use reagents. Once thawed, reporter cells can NOT be refrozen with any hope of retaining downstream assay performance. Extra volumes of these reagents should be discarded after each assay set-up.

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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp</u>
▪ PPAR α Reporter Cells	1 x 0.70 mL	-80°C
▪ PPAR δ Reporter Cells	1 x 0.70 mL	-80°C
▪ PPAR γ Reporter Cells	1 x 0.70 mL	-80°C
▪ Cell Recovery Medium 1 (CRM-1)	1 x 10.5 mL	-20°C +4°C (\leq 5 days)
▪ Compound Screening Medium 2 (CSM-2)	1 x 35 mL	-20°C +4°C (\leq 5 days)
▪ PPAR α control agonist: GW590735, 10 mM (in DMSO)	1 x 30 μ L	-20°C
▪ PPAR δ control agonist: GW0742, 1.0 mM (in DMSO)	1 x 30 μ L	-20°C
▪ PPAR γ control agonist: Rosiglitazone, 10 mM (in DMSO)	1 x 30 μ L	-20°C
▪ Detection Solution I	3 x 2.0 mL	-80°C
▪ Detection Solution II	3 x 2.0 mL	-80°C
▪ 96-well format plate frame	1	ambient
▪ snap-in, 8-well strips (white, sterile, cell culture treated)	12	ambient

III. Alternative Applications for this Reporter Assay

This PANEL of PPAR Reporter Assays is a sensitive and versatile research tool. As such, the user may configure these assays in several ways to achieve different research objectives.

The “Assay Procedure” begins in Section VI. It provides specific instructions for performing each PPAR assay, including alternative set-ups at Step 2 for the user to choose from. For example, these reporter assays may be configured to perform agonist dose-curves (Alternate 2A) or antagonist dose-curves (Alternate 2C), or to perform single-point screening of test compounds for agonist activities (Alternate 2B) or antagonist activities (Alternate 2D).

Due to the experiment-specific nature of these steps, these alternative procedures (and their attendant “NOTES”) are intended to serve as guidelines only. They are offered to assist researchers in formulating an assay design that is best suited to achieve their specific research goals.

IV. Performance Characteristics of the Individual PPAR Assays

The following figures depict the performance characteristics of the three PPAR reporter assays comprising this panel.

Figures 1-3, Part A: Representative agonist dose-response for each PPAR assay.

For each PPAR assay, eight agonist treatment concentrations, including “0” agonist, were tested. Treatment media were removed after 24 hr and LDR was applied directly to the cells. Luminescence was quantified using a Tecan GENios Pro plate-reading luminometer. Average relative light unit (RLU) values and respective standard deviations were determined. All numerical conversions and graphing were performed using GraphPad Prism software, as follows: Dose concentrations of control agonist were transformed to Log_{10} (nM). RLU values were normalized such that the lowest RLU and the highest RLU values from each data set are defined as 0% and 100%, respectively. Non-linear regression was performed using "Log (agonist) vs. normalized response - Variable slope" analyses. Error bars depict %CV. The dotted line depicts the calculated Log_{10} (EC_{50}) concentration of respective control agonists in these representative assays.

Figures 1-3, Part B: Validation of PPAR Assay performance. Signal-to-background (S/B) and Z' scores were calculated for each PPAR assay, as described by Zhang, *et al.* (1999)¹. PPAR reporter cells were treated with media alone, or media supplemented with the concentration of control ligand determined to yield RLU_{Max} . To assess the amount of background signal contributed by any other factor(s) that cause gratuitous activation of the luciferase reporter gene, “mock” reporter cells were specially prepared to contain only the luciferase vector (*i.e.*, cells without the PPAR expression vectors. Mock reporter cells are not provided with assay kits). All cells were cultured, treated with control agonist, and processed in identical manner. Luminescence measurements were conducted as described in (A). For the purposes of these analyses, RLU values are not background-subtracted.

NOTE: RLU values will vary slightly between different production lots of reporter cells, and can vary *significantly* between different makes and models of luminometers.

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.

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

Figure 1A. Agonist dose-response of the PPAR α Reporter Assay System.

Reporter cells were treated with GW590735 at the following final concentrations: 900, 300, 100, 33.3, 11.1, 3.70, 1.23, 0.412, and 0 nM.

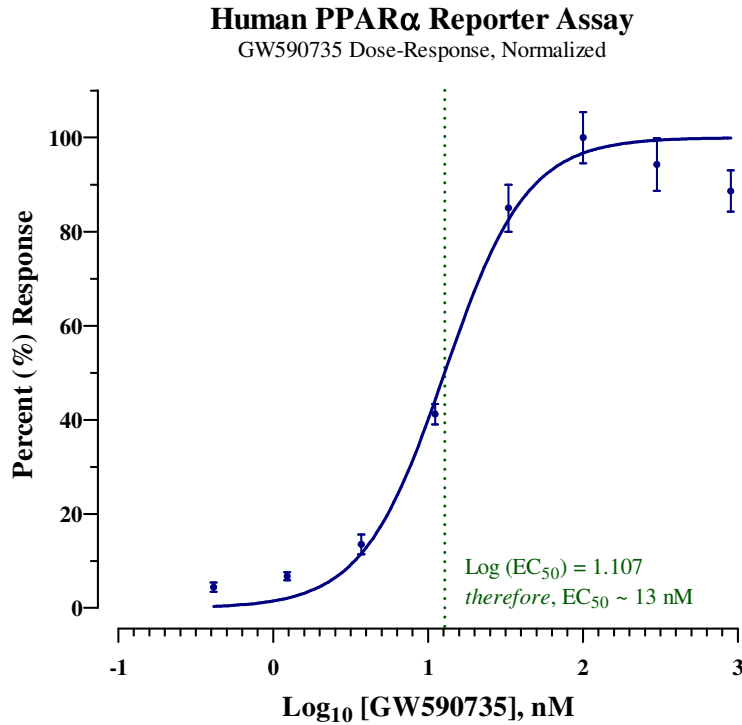


Figure 1B. Validation of the PPAR α reporter assay for screening applications.

In this particular experiment, PPAR α reporter cells treated with 300 nM GW590735 yielded S/B = 37 and a calculated Z' value of 0.75. Similarly treated mock reporter cells demonstrate only low level background luminescence ($\leq 1\%$ that of the reporter cells at EC_{Max}).

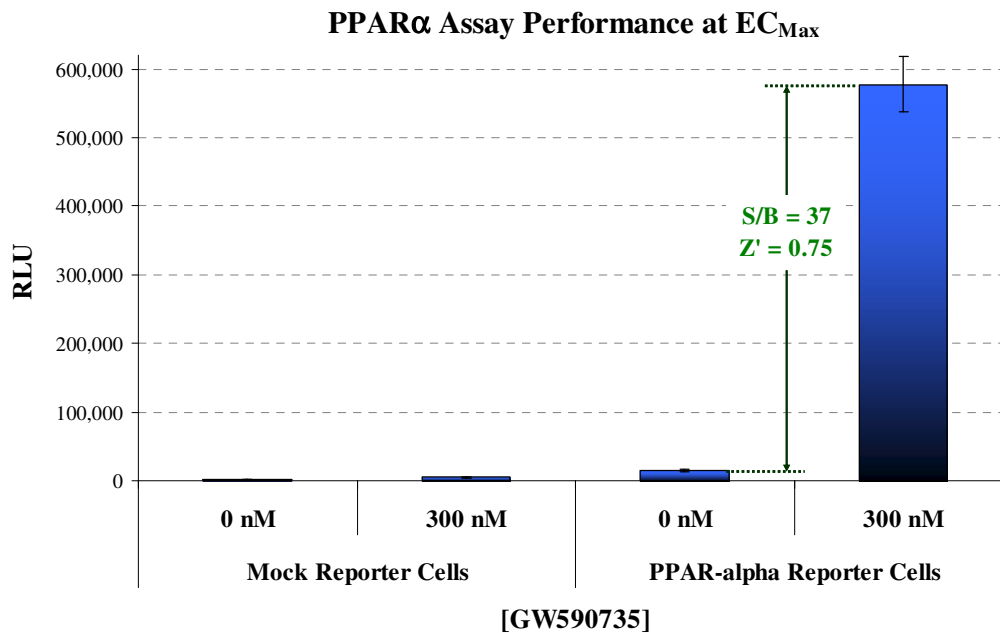


Figure 2A. Agonist dose-response of the PPAR δ Reporter Assay System.
 Reporter cells were treated with GW0742 at the following final concentrations: 90, 30, 10, 3.33, 1.11, 0.370, 0.123, 0.0411, and 0 nM.

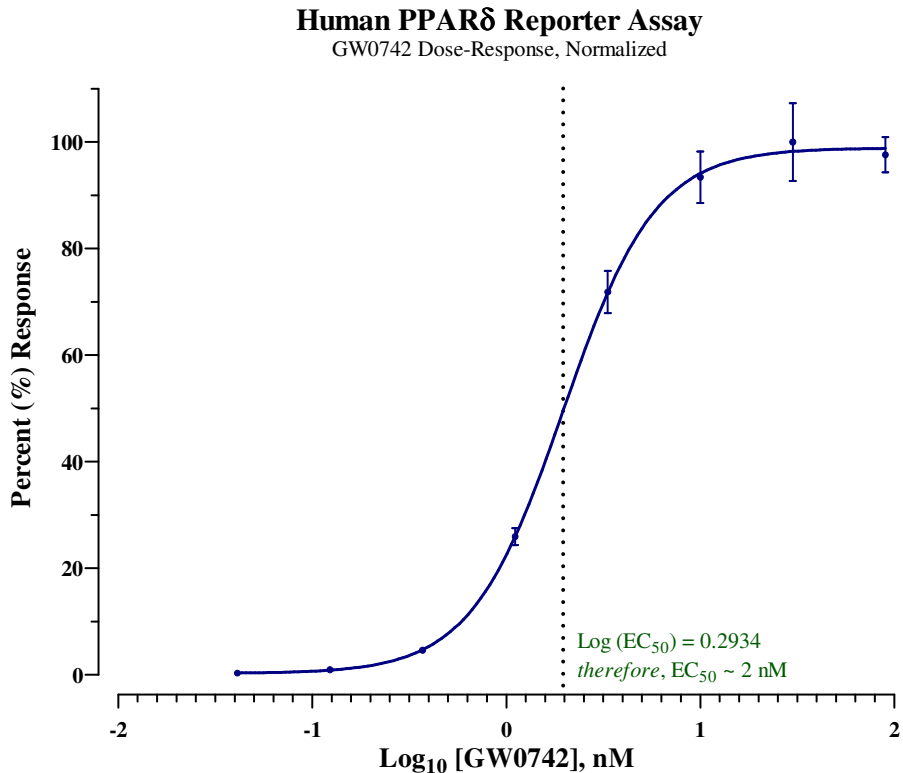


Figure 2B. Validation of the PPAR δ reporter assay for screening applications.
 In this particular experiment, PPAR δ reporter cells treated with 30 nM GW0742 yielded S/B = 520 and a calculated Z' value of 0.78. Similarly treated mock reporter cells demonstrate no significant background luminescence ($\leq 0.02\%$ that of the reporter cells at EC_{Max}).

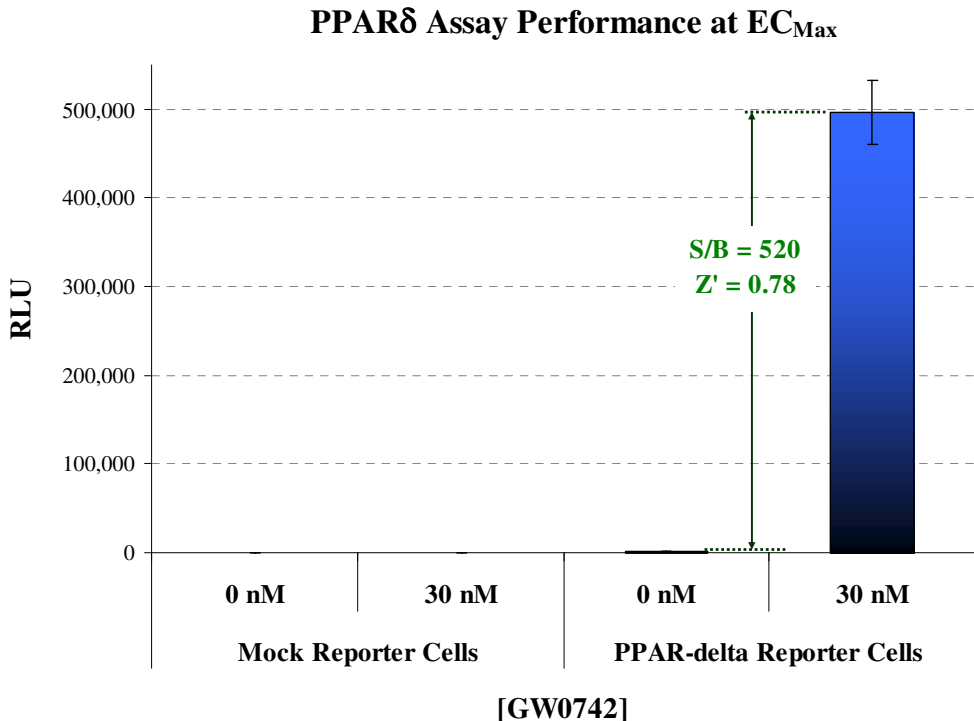


Figure 3A. Agonist dose-response of the PPAR γ Reporter Assay System.

Reporter cells were treated with Rosiglitazone at the following final concentrations: 2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6, and 0 nM.

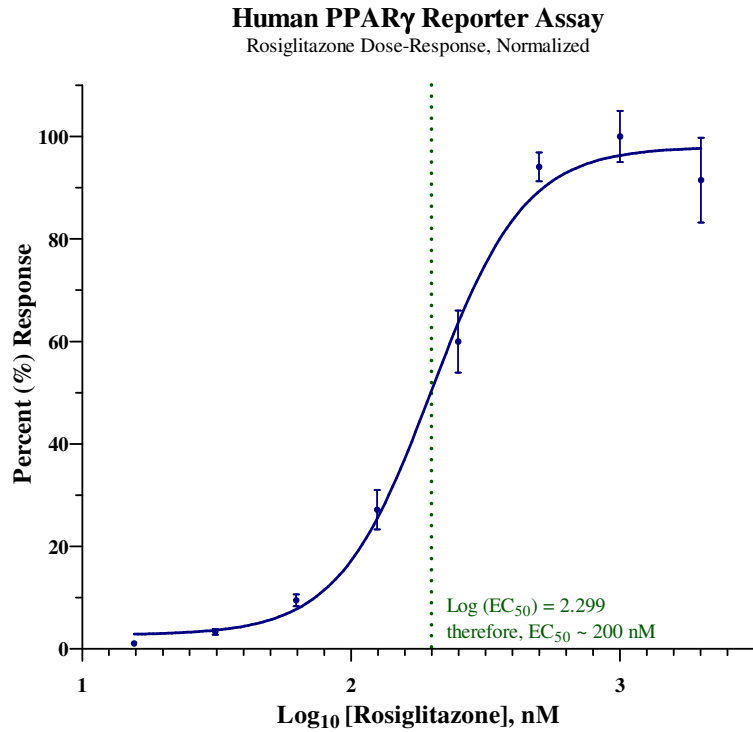
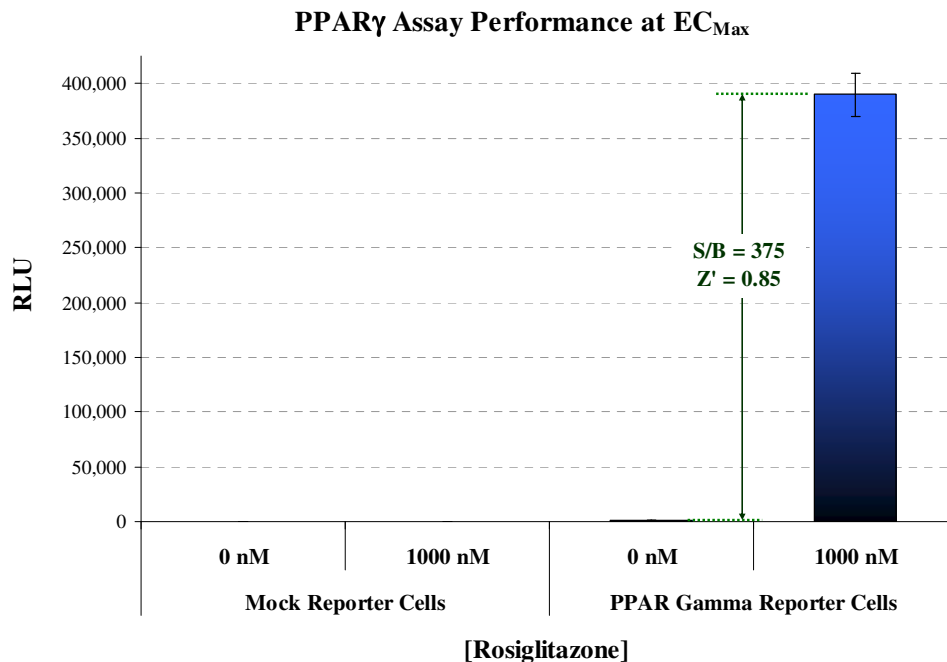


Figure 3B. Validation of the PPAR γ reporter assay for screening applications.

In this particular experiment, PPAR γ reporter cells treated with 1000 nM rosiglitazone yielded S/B = 375, and Z' value of 0.85. Similarly treated mock reporter cells demonstrate no significant background luminescence ($\leq 0.03\%$ that of the reporter cells at EC_{Max}).



V. Materials to be Supplied by the User

The following materials must be provided by the user, and should be made available prior to initiating the assay procedure:

DAY 1

- cell culture-rated hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath (*Step 3*).
- 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 control ligand(s) and user's test compound(s) (*Step 2*).
- *optional*: antagonist control compound (*Step 2*, Alternative Protocols C & D).
- 70% alcohol wipes (*Steps 3 & 4*).
- pipette & sterile tips appropriate for the transfer of 100 μ l volumes (*Steps 5 & 6*).
The use of an electronic pipette capable of repeat-dispensing is recommended.

DAY 2

- plate-reading luminometer (*Step 10*).
- a vacuum & liquid-trap apparatus connecting to a single pipette tip or, preferably, an 8-pin manifold (*e.g.*, Wheaton Science Microtest Syringe Manifold, # 851381), for use in aspirating media (*Step 12*).
- pipette and tips, as described above (*Step 13*).

VI. Assay Procedure

Review the entire assay protocol before starting.

Completion of the assay requires an overnight incubation. *Steps 1-8* are performed on **Day 1**, requiring 1-2 hours to complete. *Steps 9-15* are performed on **Day 2**, requiring ≤ 1 hour to complete.

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



- 1) Remove **Cell Recovery Medium 1 (CRM-1)** and **Compound Screening Medium 2 (CSM-2)** from freezer storage and thaw.
 - Room temperature **CSM-2** is used in the next step. A water bath may be used to facilitate rapid thawing of CSM-2.
 - Thaw **CRM-1** and warm to 37°C using a water bath. Pre-warmed CRM-1 is required in *Step 3*.



ALTERNATIVE 2A: Agonist Dose-Response Assays (as in part A of Figs. 1-3)

Use **CSM-2** to prepare a dilution series of 2x-concentrated control agonists AND an appropriate dilution series of 2x-concentrated test compound(s) to be assayed.

NOTE 2.0 In *Step 6*, 100 μ l of the prepared [CSM + test cmpd] is added into an assay well already containing 100 μ l of PPAR Reporter Cells. Therefore, to achieve the desired *final* concentration of test compound in the assay the user must prepare and dispense a “2x-concentration” of the test compound(s) (*i.e.*, [CSM + 2x test cmpd]).

NOTE 2.1 When generating dose-response curves, it is recommended to perform all measurements in triplicate. In *Step 6*, 100 μ l of [CSM + 2x test cmpd] will be added per well of the assay plate. Therefore, devise an appropriate compound dilution scheme to yield a final volume of [CSM + 2x test cmpd] that is slightly greater than 300 μ l, thus allowing accurate volume transfers into each of the triplicate wells of the assay plate.

(Continued on p. 12)

NOTE 2.2 Hints about making appropriate dilutions of control agonists:

- **PPAR α** When using GW590735 as the positive-control agonist for PPAR α , we find the following assay concentration range provides a complete dose-response: 900, 300, 100, 33.3, 11.1, 3.70, 1.23, 0.412 and 0 nanoMolar (nM; 10^{-9} Molar), as depicted in **Figure 1A**. However, as explained in *Note 2.0*, a 2x-concentrated dilution series of both the positive-control and test compounds are required. Generating this dilution series for GW590735 may be achieved by following the example presented in **APPENDIX 1A**. In brief, use a portion of CSM to first perform a 55.5-fold dilution of the provided **10 mM** GW590735 stock. Use this intermediate stock solution to perform a subsequent 100-fold dilution to achieve the first desired 2x-concentration of 1800 nM. Continue by using a portion of the 1800 nM solution to perform seven sequential 3-fold dilutions to produce 600, 200, 66.7, 22.2, 7.41, 2.47, and 0.823 nM 2x-concentrated stocks. Neat CSM (or CSM supplemented with 0.018% DMSO; *i.e.*, the highest concentration of "vehicle") may be used to provide the "0 nM" control treatment.]
- **PPAR δ** When using GW0742 as the positive-control agonist for PPAR δ , we find the following assay concentration range provides a complete dose-response: 90, 30, 1.0, 3.33, 1.11, 0.370, 0.123, 0.412 and 0 nM, as depicted in **Figure 2A**. However, as explained in *Note 2.0*, a 2x-concentrated dilution series of both the positive-control and test compounds are required. Generating this dilution series for GW0742 may be achieved by following the example presented in **APPENDIX 1B**. In brief, use a portion of CSM to first perform a 55.5-fold dilution of the provided **1.0 mM** GW0742 stock. Use this intermediate stock solution to perform a subsequent 100-fold dilution to achieve the first desired 2x-concentration of 180 nM. Continue by using a portion of the 180 nM solution to perform seven sequential 3-fold dilutions to produce 60, 20, 6.67, 2.22, 0.741, 0.247, and 0.0823 nM 2x-concentrated stocks. Neat CSM (or CSM supplemented with 0.018% DMSO; *i.e.*, the highest concentration of "vehicle") may be used to provide the "0 nM" control treatment.]
- **PPAR γ** When using rosiglitazone as the positive-control agonist for PPAR γ , we find the following assay concentration range provides a complete dose-response: 2000, 1000, 500, 250, 125, 62.5, 31.3, 15.6 and 0 nM, as depicted in **Figure 3A**. However, as explained in *Note 2.0*, a 2x-concentrated dilution series of both the positive-control and test compounds are required. Generating this dilution series for rosiglitazone may be achieved by following the example presented in **APPENDIX 1C**. In brief, use a portion of CSM to first perform a 50-fold dilution of the provided **10 mM** rosiglitazone stock. Use this intermediate stock solution to perform a subsequent 50-fold dilution to achieve the first desired 2x-concentration of 4000 nM. Continue by using a portion of the 4000 nM solution to perform seven sequential 2-fold dilutions to produce 2000, 1000, 500, 250, 125, 62.5 and 31.3 nM 2x-concentrated stocks. Neat CSM (or CSM supplemented with 0.04% DMSO; *i.e.*, the highest concentration of "vehicle") may be used to provide the "0 nM" control treatment.]

ALTERNATIVE 2B: Screening for Agonist Activities via Single-Point Assay

Use **CSM-2** to make an appropriate dilution of each test compound in the library. Also, prepare CSM-2 containing the desired positive-control reference agonist.

This PPAR Reporter Assay PANEL includes:

- **10 mM** stock solution of **GW590735**, an agonist of **PPAR α** that may be used as a positive-control. An assay concentration of 300 nM GW590735 typically provides $\geq 95\%$ activation of PPAR α (Figure 1A). Hence, 600 nM GW590735 will provide a 2x-concentrated agonist that is a suitable positive-control reference. Refer to *Note 2.2* for a recommended dilution scheme to prepare the appropriate 2x-concentrated GW590735 solution.
- **1.0 mM** stock solution of **GW0742**, an agonist of **PPAR δ** that may be used as a positive-control. An assay concentration of 30 nM GW0742 typically provides $\geq 95\%$ activation of PPAR δ (Figure 2A). Hence, 60 nM GW0742 will provide a 2x-concentrated agonist that is a suitable positive-control reference. Refer to *Note 2.2* for a recommended dilution scheme to prepare the appropriate 2x-concentrated GW0742 solution.
- **10 mM** stock solution of **Rosiglitazone**, an agonist of **PPAR γ** that may be used as a positive-control. An assay concentration of 1000 nM Rosiglitazone typically provides $\geq 95\%$ activation of PPAR γ (Figure 3A). Hence, 2000 nM Rosiglitazone will provide a 2x-concentrated agonist that is a suitable positive-control reference. Refer to *Note 2.2* for a recommended dilution scheme to prepare the appropriate 2x-concentrated rosiglitazone solution.

NOTE 2.3 As a general rule, when an organic solvent is used to generate primary stock solutions of test compounds, we recommend that the user devise a dilution scheme so that the concentration of organic solvent in [CSM + 2x test cmpd] does not exceed 0.2% (i.e., minimally a 500-fold dilution into CSM). This corresponds to a final assay concentration of 0.1% organic solvent.

NOTE 2.4 In *Step 6*, 100 μ l of the prepared [CSM + 2x test cmpd] will be added per well of the assay plate. If single-point assays are to be made, prepare a volume of each [CSM + 2x test compound] media that is slightly greater than 100 μ l, thus allowing accurate volume transfers into respective assay wells. This assay kit provides 35 ml of CSM to be used in preparing dilutions of control and test compounds for all 96 assays. Plan dilution schemes *carefully*.

ALTERNATIVE 2C: Antagonist Dose-Response Assays

A common method of performing receptor inhibition studies is to prepare a co-mix of a known agonist (at a constant concentration typically between EC₅₀ – EC₈₅) AND a dilution series of the test antagonist compound(s) to be evaluated.

See *NOTE 2.0*.

This PPAR Assay PANEL includes PPAR-specific control agonists that may be used to setup receptor inhibition studies. Suggested concentrations for respective assays are below. Refer to *NOTE 2.2* for dilution schemes to aid in the preparing appropriate [CSM + 2x control agonist] treatment media.

- **GW590735**, an agonist of **PPAR α** (Figure 1A). We find that 20 nM GW590735 typically approximates EC₇₀ in this reporter assay.
- **GW0742**, an agonist of **PPAR δ** (Figure 2A). We find that 3.3 nM GW0742 typically approximates EC₇₅ in this reporter assay.
- **Rosiglitazone**, an agonist of **PPAR γ** (Figure 3A). We find that 300 nM Rosiglitazone typically approximates EC₇₀ in this reporter assay.

Supplement a portion of **CSM-2** with an appropriate volume of agonist to generate a sufficient stock volume of [CSM + 2x agonist]. Use this [CSM + 2x agonist] stock to then prepare a 2x-concentrated dilution series of each test compound to be evaluated for antagonist activity. If desired, also prepare a dilution series of 2x-concentrated positive-control antagonist (supplied by the user).

NOTE 2.5 When generating antagonist dose-response curves, it is recommended to perform all measurements in triplicate. In *Step 6*, 100 μ l of [CSM + 2x agonist + 2x test cmpd] will be added per well of the assay plate. Therefore, devise an appropriate compound dilution scheme to yield a final volume of [CSM + 2x agonist + 2x test cmpd] that is slightly greater than 300 μ l, thus allowing accurate volume transfers into each of the triplicate wells of the assay plate.

NOTE 2.6 As a *general rule*: when an organic solvent is used to generate primary stock solutions of test compounds, we recommend that the user devise a dilution scheme so that the concentration of organic solvent in [CSM + 2x agonist + 2x test cmpd] does not exceed 0.2% (*i.e.*, *minimally* a 500-fold dilution into CSM). This corresponds to a final assay concentration of 0.1% organic solvent.

ALTERNATE 2D: Screening for Antagonist Activities via Single-Point Assay

A common method of performing receptor inhibition studies is to prepare a co-mixture of a known agonist (at a concentration typically between EC₅₀ – EC₈₅) AND a single test concentration of the candidate antagonist compounds to be evaluated. As described in ALTERNATE 2C, this PPAR Assay PANEL includes agonist control compounds for each of the 3 PPAR assays in this panel. These compounds may be used to design such receptor inhibition screens.

See *NOTE 2.0*.

Supplement a portion of **CSM-2** with an appropriate volume of agonist to generate an appropriate stock volume of [CSM + 2x agonist]. Refer to *NOTE 2.2* for a recommended dilution scheme to prepare [CSM + 2x agonist]. Use this stock of [CSM + 2x agonist] to then prepare a 2x-concentrated dilution of each test compound to be screened for antagonist activity. If desired, also prepare an appropriate dilution of 2x-concentrated positive-control antagonist (supplied by the user).

See *NOTE 2.6*.

NOTE 2.7 In *Step 6*, 100 µl of the prepared [CSM + 2x agonist + 2x test cmpd] will be added per well of the assay plate. If single-point assays are to be made, prepare a volume of each [CSM + 2x agonist + 2x test cmpd] media that is slightly greater than 100 µl, thus allowing accurate volume transfers into respective assay wells. This assay kit provides 35 ml of CSM to be used in preparing dilutions of control and test compounds for all 96 assays. Plan dilution schemes carefully!

- 3) Retrieve the **PPAR Reporter Cells** from -80°C storage. Retrieve **CRM-1** from the 37°C water bath and sanitize the outside of the tube with a 70% ethanol swab. Transfer the tubes into a cell-culture hood. Perform a *rapid thaw* of the frozen cells by pipette-transferring 3.0 ml of the 37°C CRM-1 into the tube of frozen cells. Recap the tubes of Reporter Cells and immediately place them in a 37°C water bath for at least 3 minutes.

NOTE: During this incubation step, work in an aseptic hood to *carefully* mount the appropriate number of sterile 8-well strips into the blank plate frame. Each PPAR assay utilizes 4x 8-well strips, for a total of 32 reactions each. Strip-wells are fragile. Note that they have *keyed ends* (square and round), hence, they will fit into the frame in only one orientation.

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

- 5) Gently invert the tubes of Reporter Cells to disperse any cell aggregates. For each PPAR assay, dispense 100 µl of a given cell suspension into **32 wells** of the **Assay Plate**.

NOTE: Take care to prevent cells from settling during the dispensing period. Lack of precision in transferring uniform volumes across the assay plate, and/or allowing cells to settle during the dispensing process, will cause well-to-well variation in the assay. For improved speed, precision, and ergonomic comfort, *the use of an electronic repeat-dispensing pipette is recommended.*

- 6) Add 100 µl of 2x-concentrated treatment media (prepared in *Step 2*) to appropriate wells of the assay plate.

- 7) Replace the plate's lid and transfer it into a 37°C, humidified 5% CO₂ incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity environment within the cell culture incubator. This is necessary to prevent the onset of deleterious "edge-effects" in the assay plate.

- 8) For greater convenience on Day 2, retrieve **Detection Solutions I and II** from -80°C storage and place in a dark refrigerator (4°C) to thaw overnight.

NOTE: This PANEL kit contains three aliquots of Detection Solution I, and three aliquots of Detection Solution II. These solutions are provided in 3 discreet pairs so that, if preferred, users may perform individual PPAR assays at different times. Thaw 1 pair of Detection Solutions I & II for each PPAR assay. If the user intends to perform all three PPAR assays at the same time, place all three pairs of Detection Solutions in a refrigerator to thaw overnight. In such cases, on **Day 2** (*Step 11*) combine all of the separate solutions into one tube to generate a single batch volume of the final **LDR** mixture.

(Continue on DAY 2)

DAY 2: Subsequent manipulations do not require special regard for aseptic technique.

- 9) 30 minutes before intending to quantify PPAR γ activity, remove **Detection Solutions I and II** from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature. Gently invert each tube several times to ensure homogenous solutions.
- NOTE:* Do NOT actively warm Detection Solutions I and II 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) Turn on the luminometer. Set the instrument to perform a single 5 second “plate shake” prior to reading the assay plate. Set the read time for 0.5 second per well.
- NOTE:* Many luminometers require a “warm up” period of 15 minutes *or more* before the photo-multiplier tube (PMT) attains maximal precision in reading very low luminescence signal.
- 11) *Immediately before proceeding to Step 12*, combine the contents of Detection Solutions I and II into a single tube to generate **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.
- 12) After 22-24 hours of incubation, remove the assay plate from the incubator. Remove the plate’s lid. Remove media contents from each well.
- NOTE:* 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. Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media *via* a sweeping downward movement is NOT advised.
- 13) Add 100 μ l of room temperature **LDR** to each well of the assay plate.
- NOTE:* *Pipette carefully to avoid bubble formation!* Scattered micro-bubbles will not pose a problem. However, bubbles covering the surface of the reaction mix, or large bubbles clinging to the side walls of the well, will cause lens-effects that may significantly degrade the accuracy and precision of the assay data. In the event of excessive bubble formation during processing, spin the assay plate (with lid) at *low speed* for 1-2 minutes using a room temperature centrifuge fitted with counter-balanced plate carriers.
- 14) Allow the assay plate to rest at room temperature for at least 15 minutes after the addition of LDR. Do not shake the assay plate during this period.
- NOTE:* See **APPENDIX 2** for information regarding signal stability.
- 15) Read the assay plate anytime between 15 - 90 minutes after adding LDR.

VI. Related Products

PPARα Family of Assay Products	
<i>Product No.</i>	<i>Product Descriptions</i>
IB00111-32	Human PPAR α Reporter Assay System 3x 32 assays in 96-well format
IB00111	Human PPAR α Reporter Assay System 1x 96-well format assay
IB00111-B10	Human PPAR α Reporter Assay System (Bulk Pack) Bulk Reagent Pack for HTS in 96-well format
IB00112	Human PPAR α Reporter Assay System 1x 384-well format assays
IB00112-B10	Human PPAR α Reporter Assay System (Bulk Pack) Bulk Reagent Pack for HTS in 384-well format
PPARδ Family of Assay Products	
IB00121-32	Human PPAR δ Reporter Assay System 3x 32 assays in 96-well format
IB00121	Human PPAR δ Reporter Assay System 1x 96-well format assay
IB00121-B10	Human PPAR δ Reporter Assay System (Bulk Pack) Bulk Reagent Pack for HTS in 96-well format
IB00122	Human PPAR δ Reporter Assay System 1x 384-well format assays
IB00122-B10	Human PPAR δ Reporter Assay System (Bulk Pack) Bulk Reagent Pack for HTS in 384-well format
PPARγ Family of Assay Products	
IB00101-32	Human PPAR γ Reporter Assay System 3x 32 assays in 96-well format
IB00101	Human PPAR γ Reporter Assay System 1x 96-well format assays
IB00101-B10	Human PPAR γ Reporter Assay System (Bulk Pack) Bulk Reagent Pack for HTS in 96-well format
IB00102	Human PPAR γ Reporter Assay System 1x 384-well format assays
IB00102-B10	Human PPAR γ Reporter Assay System (Bulk Pack) Bulk Reagent Pack for HTS in 384-well format
Alternative volumes of each PPAR Assay's Bulk Reagents can be custom manufactured. Please Inquire.	

Please refer to INDIGO Biosciences' website for updated product offerings.

www.indigobiosciences.com

VII. Limited Use Disclosures

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The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Patent 5,583,024. INDIGO Biosciences, Inc. has entered into a license agreement with The Regents of the University of California (Oakland, CA) for commercial application of the cDNA encoding the native luciferase of *Photinus pyralis*.

The method of recombinant expression of the human PPAR γ is a patented technology. INDIGO Biosciences, Inc. has entered into a license agreement with the assignees that mandates the following disclosure:

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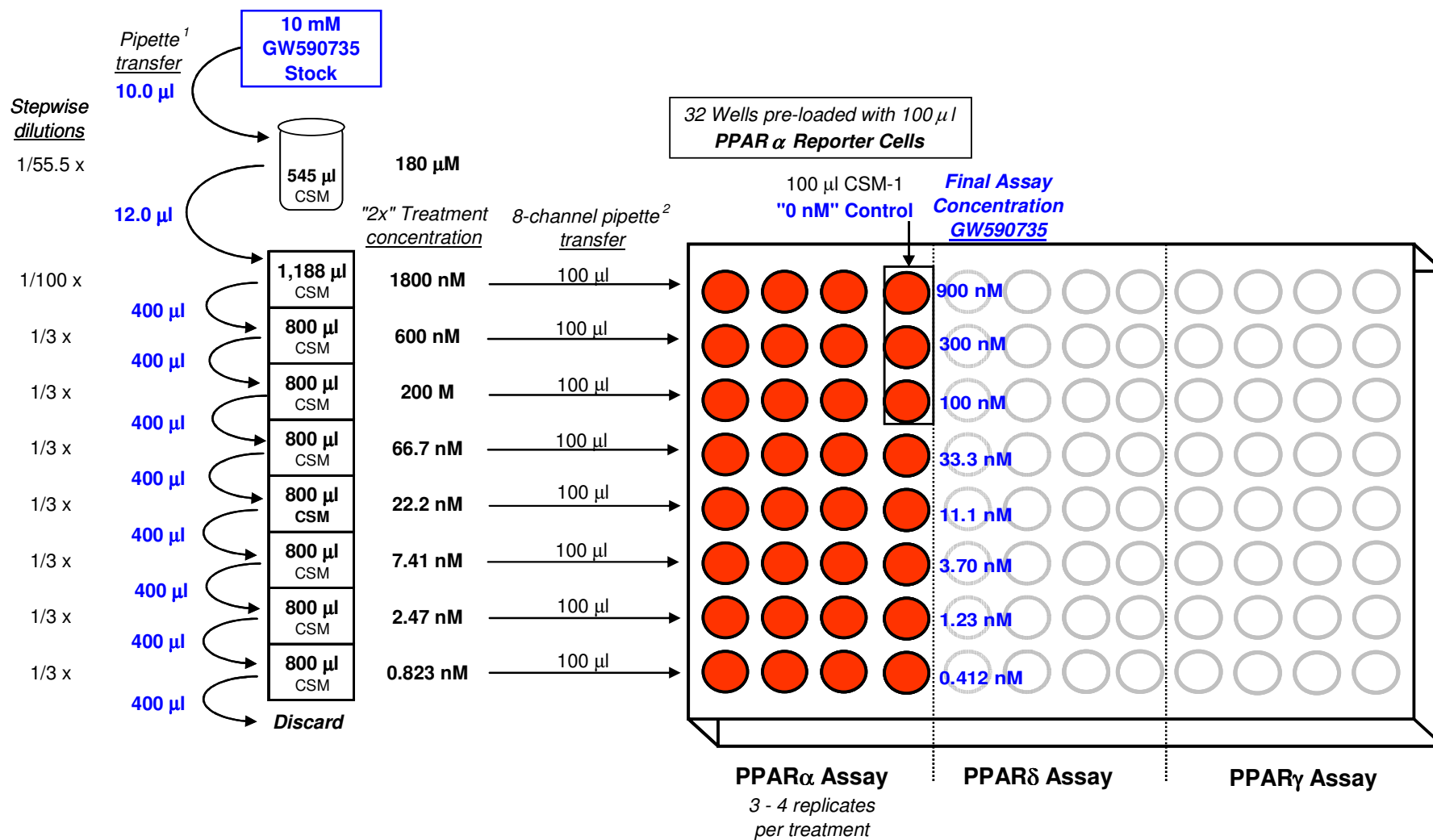
“CryoMite” is a Trademark TM of INDIGO Biosciences, Inc.

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APPENDIX 1A

Example Dilution Scheme for GW590735 & Setup of PPAR α Control Agonist Dose-Response Assay

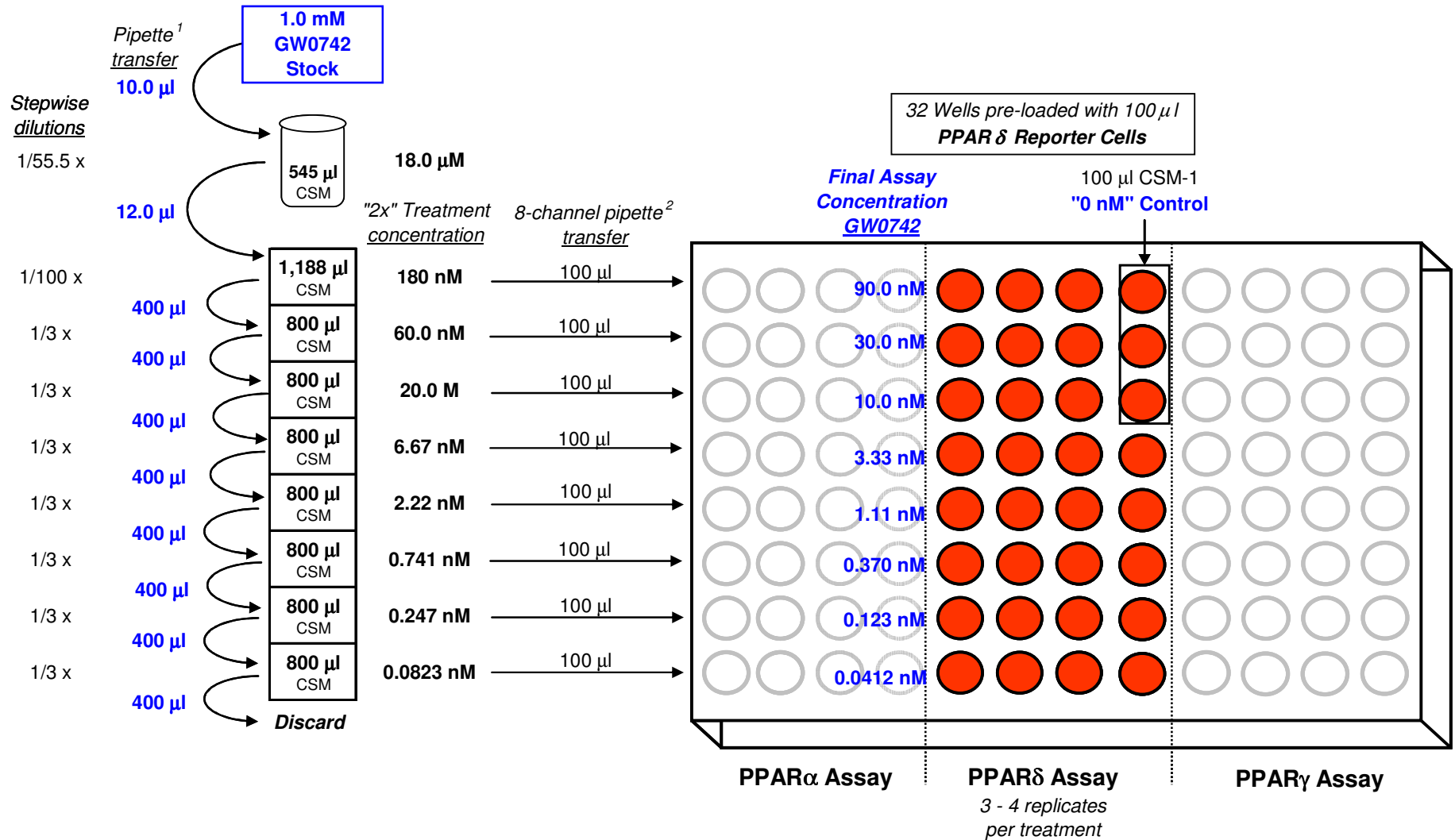


¹ To maximize accuracy, use calibrated pipettes that most closely match the desired transfer volume. For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.

² To maximize speed, precision, and ergonomic comfort during manual pipetting of 100 μ l volumes of treatment media, the use of an electronic, 8-channel, multi-dispensing P1000 pipette is recommended.

APPENDIX 1B

Example Dilution Scheme for GW0742 & Setup of PPAR δ Control Agonist Dose-Response Assay

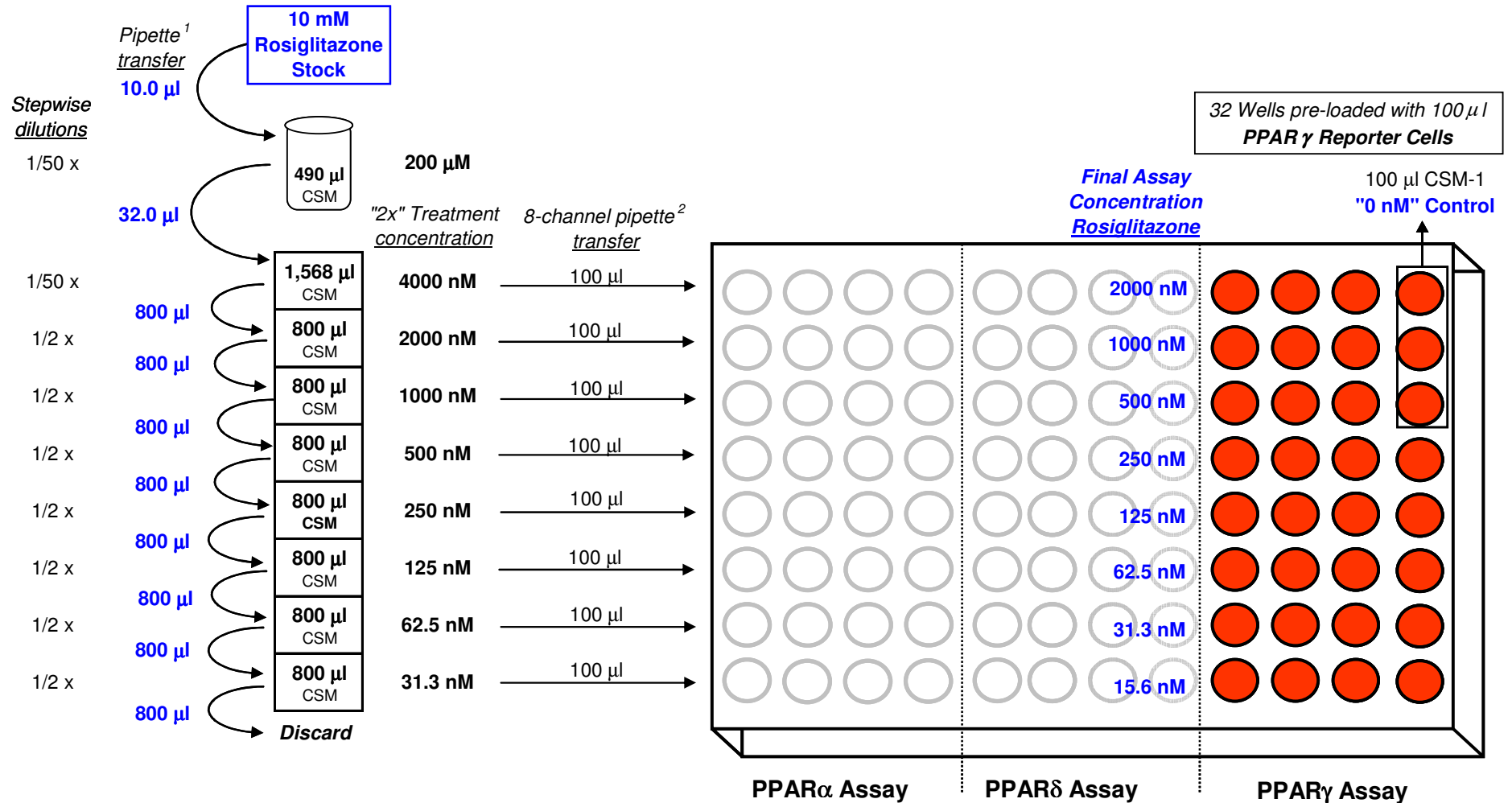


¹ To maximize accuracy, use calibrated pipettes that most closely match the desired transfer volume. For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.

² To maximize speed, precision, and ergonomic comfort during manual pipetting of 100 μl volumes of treatment media, the use of an electronic, 8-channel, multi-dispensing P1000 pipette is recommended.

APPENDIX 1C

Example Dilution Scheme for Rosiglitazone & Setup of PPAR γ Control Agonist Dose-Response Assay



¹ To maximize accuracy, use calibrated pipettes that most closely match the desired transfer volume. For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.

² To maximize speed, precision, and ergonomic comfort during manual pipetting of 100 μ l volumes of treatment media, the use of an electronic, 8-channel, multi-dispensing P1000 pipette is recommended.

APPENDIX 2

Signal Stability of the Nuclear Receptor Reporter Assay

The human ER β Reporter Assay System is used here to demonstrate the light emission profile characteristic of INDIGO Biosciences' nuclear receptor assay system products.

As seen in **FIGURE 3**, between 5 and 15 minutes after adding LDR to assay wells the initial intensity of luminescence decays by 10-12%. However, luminescence signal stabilizes and remains essentially constant over the ensuing 75 minute reaction period. From T=15 minute to T=90 minutes, average luminescence measured from the same set of assay wells deviate by less than 5%

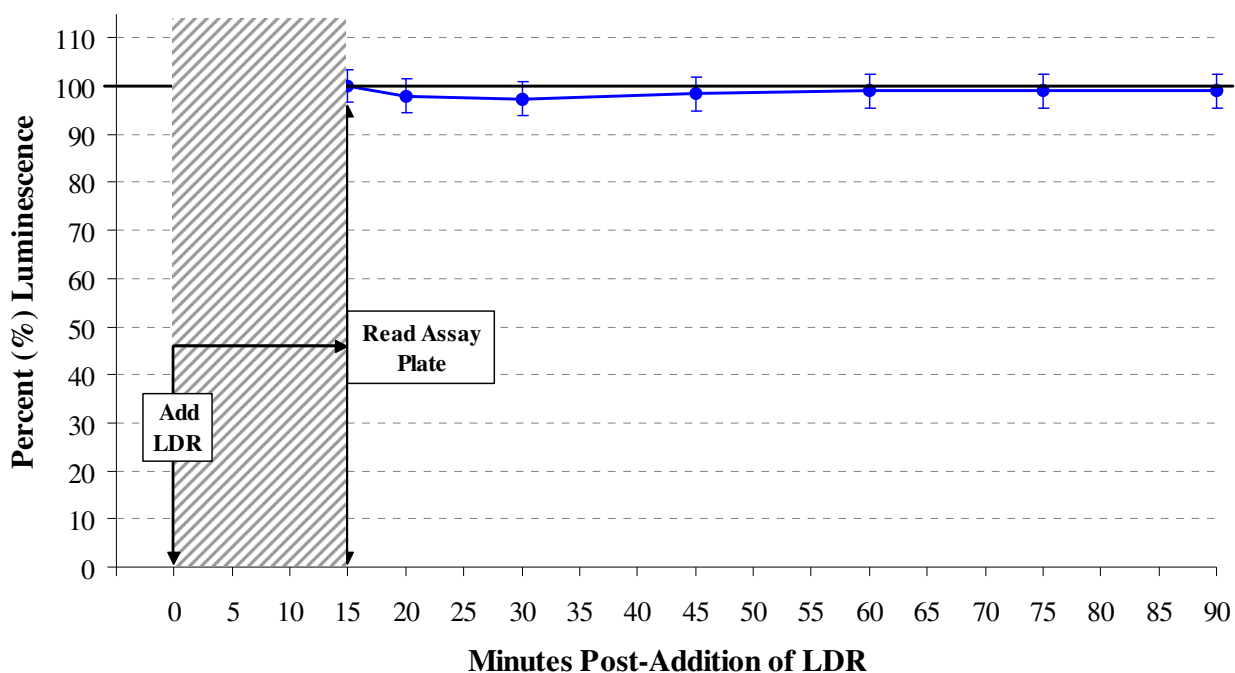


Figure 3. Stability of the luminescence signal. ER β reporter cells were cultured in 8 replicate wells of a 96-well assay plate, treated with 1 nM 17- β -estradiol for 24 hr, and media were replaced with LDR according to the protocol provided in Technical Manual #00411. Following an initial 5 minute rest period at room temperature, luminescence intensities were quantified by integrating light emission over 500 mSec. After the initial 5 minute time point, the assay plate was re-read at 10, 15, 20, 30, 45, 60, 75 and 90 minutes post-addition of LDR. Average RLU and respective standard deviation values were calculated, then normalized so that the luminescence signal at 15 minutes = 100%.

Allowing a *minimum* rest period of 15 minutes after the addition of LDR is particularly important for HTS users. Due to the logistics of batch-processing large numbers of assay plates, a significant time differential may occur between processing the *first* and *last* assay plates. Nonetheless, due to the stable emission profile of the luciferase reaction between 15 - 90 minutes, HTS users may be confident in comparing signal output from test samples in the first assay plate to those in the last plate in the stack.