

**Human Pregnane X Receptor  
(NR1I2, PXR, SXR)  
Reporter Assay System**

**96-well Format Assays**  
Product # IB07001

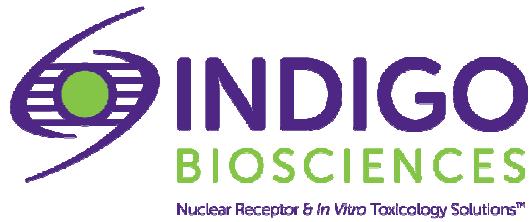
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**Technical Manual**  
(version 7.2i)

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## **Human PXR Reporter Assay System 96-well Format Assays**

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## **I. Description**

### **▪ The Assay System ▪**

This assay utilizes proprietary human cells engineered to provide constitutive, high-level expression of the **Human Pregnane X Receptor** (NR1I2), a ligand-dependent transcription factor commonly referred to as **PXR**. PXR is also known as the Steroid and Xenobiotic sensing nuclear receptor (**SXR**).

INDIGO's Reporter Cells express a hybrid form of human PXR. The N-terminal sequence encoding the PXR DNA binding domain (DBD) has been substituted with that of the yeast GAL4-DBD. The native PXR ligand binding domain (LBD) and other C-terminal domains remain intact and functional. Ligand interaction activates the receptor, causing it to bind to the GAL4 DNA binding sequence, which is functionally linked to a resident luciferase reporter gene. Thus, quantifying changes in luciferase activity in the treated reporter cells provides a sensitive surrogate measure of the changes in PXR activity. The principal application of this assay kit is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against human PXR.

PXR 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 or viability determinations prior to assay setup.

INDIGO Bioscience's assays are all-inclusive cell-based assay systems. In addition to PXR Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, the reference agonist Rifampicin, Luciferase Detection Reagent, and a cell culture-ready assay plate.

### **▪ The Assay Chemistry ▪**

INDIGO's cell-based 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<sup>+2</sup>-dependent reaction that consumes O<sub>2</sub> and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP<sub>i</sub>, CO<sub>2</sub>, 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 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 ▪

Small molecule test compounds are typically solvated in DMSO at high concentrations; ideally 1,000x-concentrated stocks relative to the highest desired treatment concentration in the assay. Using high-concentration stocks minimizes DMSO carry-over into the assay plates. Immediately prior to setting up an assay, the master stocks are serially diluted using one of two alternative strategies:

1.) As described in *Step 7* and depicted in Appendix 1 for the reference agonist, **Compound Screening Medium (CSM)** may be used as the diluent to make serial dilutions of test compounds to achieve the desired final assay concentration series.

Alternatively, if test compound solubility is expected to be problematic,

2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

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

**NOTE:** CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup and are considered to be 'single-use' reagents.

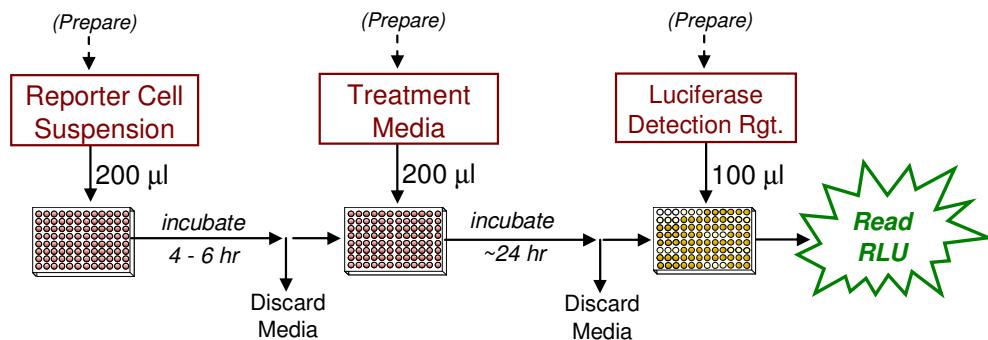
## ▪ Considerations for Automated Dispensing ▪

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.

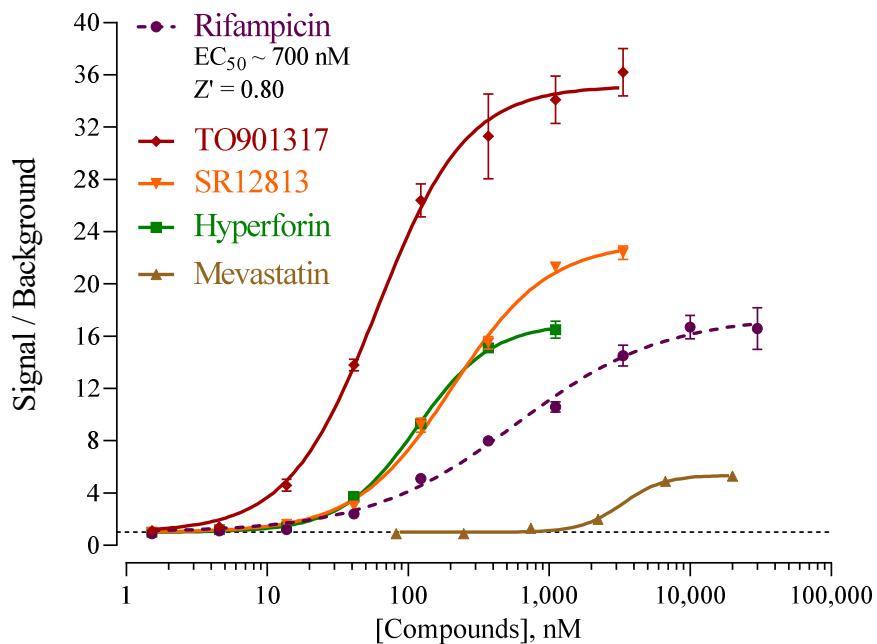
Stock Reagent & Volume provided	Volume to be Dispensed (96-well plate)	Excess rgt. volume available for instrument dead volume
<b>Reporter Cell Suspension</b> 21 ml (prepared from kit components)	200 $\mu$ l / well 19.2 ml / plate	~ 1.8 ml
<b>LDR</b> 12 ml (prepared from kit components)	100 $\mu$ l / well 9.6 ml / plate	~ 2.4 ml

## ▪ Assay Scheme ▪

**Figure 1.** Assay workflow. *In brief*, 200  $\mu$ l of Reporter Cells is dispensed into wells of the assay plate and pre-incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200  $\mu$ l/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 ▪  
**Human PXR (NR1I2) Agonist Assays**



**Figure 2. Agonist dose-response analyses of Human PXR.**

Performance of the human PXR assay using the reference agonists Rifampicin (provided), Hyperforin dicyclohexylammonium (Enzo Life Sciences), TO901317(Cayman Chemical), SR12813 (Tocris), and Mevastatin (Cayman Chemical). Luminescence was quantified and average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration ( $n = 4$ ). Fold-activation and  $Z'$  values were calculated as described by Zhang, *et al.* (1999)<sup>1</sup>. Non-linear regression and  $EC_{50}$  analyses were performed using GraphPad Prism software. High  $Z'$  scores confirm the robust performance of this assay, and its suitability for HTS<sup>1</sup>.

<sup>1</sup> 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.

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

## ***II. Product Components & Storage Conditions***

This Human PXR Assay kit contains materials to perform assays in a single 96-well assay plate.

**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.

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.

<b><i>Kit Components</i></b>	<b><i>Amount</i></b>	<b><i>Storage Temp.</i></b>
▪ PXR Reporter Cells	1 x 2.0 mL	<b>-80°C</b>
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ Rifampicin, 30 mM (in DMSO) (reference agonist for PXR)	1 x 30 µL	-20°C
▪ Detection Substrate	1 x 6.0 mL	<b>-80°C</b>
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well, <i>collagen-coated</i> assay plate (white, sterile, cell-culture ready)	1	<b>-20°C</b>

*NOTE:* This Assay kit contains one 96-well assay plate in which the assay wells have been collagen-coated and dried; the assay plate should be stored frozen (-20°C or colder) until use.

## ***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<sub>2</sub> 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:* clear 96-well assay plate, 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 1-11* are performed on **Day 1**, requiring less than 2 hours of actual bench work plus a 4 hr pre-incubation step. *Steps 12-17* 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<sub>50</sub> – EC<sub>85</sub>) of a known agonist AND varying concentrations of the test compound(s) to be evaluated for antagonist activity. This PXR Assay kit includes a 30 mM stock solution of **Rifampicin**, a low-potency agonist of PXR that may be used to setup antagonist-mode assays. 3  $\mu$ M Rifampicin typically approximates EC<sub>70-80</sub> in this cell-based assay. Hence, it presents a suitable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

Add the challenge agonist (rifampicin) to a bulk volume of **CSM** at an EC<sub>50</sub> – EC<sub>85</sub> concentration. This medium is then used to prepare serial dilutions of test compounds to achieve the desired respective final assay concentrations. This is an efficient and precise method for setting up PXR antagonist assays, and it is the method presented in *Step 7b* of this protocol.

**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 the tube of **Reporter Cells** from -80°C storage, place it directly into dry ice and transport the cells to the laminar flow hood. When ready to begin, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by transferring 9.5 ml from **each of the 2 tubes** of 37°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°C water bath for 5 minutes. The resulting volume of cell suspension will be **21 ml**.

**3.) Retrieve the tube of Reporter Cell Suspension** from the water bath and sanitize the outside surface with a 70% alcohol swab.

**4.) Gently invert the tube of Reporter Cells** several times to gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an 8-chanel pipette, dispense **200  $\mu$ l / well** of cell suspension into the assay plate.

*NOTE 4.1:* If INDIGO's Live Cell Multiplex Assay is to be incorporated then 'cell blank' wells (meaning cell-free, but containing 'CSM') must be included to allow quantification of plate-specific fluorescence background (refer to the LCMA Technical Manual).

*NOTE 4.2:* 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 4.3:* 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, preferably collagen-coated, 96-well assay plate. Continue to process this plate in identical manner to the white assay plate.

**5.) Pre-incubate reporter cells:** Place the assay plate into a 37°C, ≥ 70% humidity, 5% CO<sub>2</sub> incubator for 4 - 6 hours.

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

**7.) 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 9*, 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 organic solvent) carried over into assay reactions should not exceed 0.4%.

**Agonist-mode assays.** This Assay kit includes a 30 mM stock solution of Rifampicin, the most commonly cited reference agonist of human PXR. The following 7-point treatment series, prepared in serial 3-fold decrements, provides a complete dose-response: 30.0, 10.0, 3.33, 1.11, 0.370, 0.123 and 0.0412µM. Be sure to include 'no treatment', or 'vehicle only', control wells. **APPENDIX 1** provides an example for generating such a dilution series.

~ or ~

**b. Antagonist-mode assays.** When setting up antagonist assays, first supplement a bulk volume of CSM with the challenge agonist (rifampicin) to achieve the desired final assay-concentration (refer to "*A word about antagonist-mode assay setup*", pg. 7). The agonist-supplemented CSM is then used to generate dilutions of test compound stocks to achieve their final assay concentrations.

**8.)** At the end of the 4-6 hours pre-culture period, discard the media. The preferred method is to use a 'wrist flick' to manually eject media 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.

**9.) Dispense 200 µl / well** of each prepared treatment media into the assay plate.

*Hint:* If well-to-well variation due to 'edge-effects' is a concern this problem may be mitigated by dispensing sterile liquid into the *inter-well* spaces of the assay plate. Simply remove 1 tip from the 8-channel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

**10.) 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.

**11.)** 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.

**12.)** 30 minutes before intending to quantify receptor 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.

*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.

**13.)** 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*.

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

**15.)** Following 22 - 24 hours incubation in treatment media, discard the 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.

**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 -10 minutes following the addition of LDR. Do not shake the assay plate during this period.

**17.)** Quantify luminescence.

## **V. Related Products**

<i>Product No.</i>	<i>Product Descriptions</i>
<b>Human PXR Assay Kit Products</b>	
IB07001-32	3x 32 Human PXR assays; strip-wells in 96-well plate frame
IB07001	1x 96-well format Human PXR assays
IB07002	1x 384-well format Human PXR assays
<b>Rat PXR Assay Kit Products</b>	
R07001-32	3x 32 Rat PXR assays; strip-wells in 96-well plate frame
R07001	1x 96-well format Rat PXR assays
<b>Mouse PXR Assay Kit Products</b>	
M07001-32	3x 32 Mouse PXR assays; strip-wells in 96-well plate frame
M07001	1x 96-well format Mouse PXR assays
<b>Dog PXR Assay Kit Products</b>	
D07001-32	3x 32 Dog PXR assays; strip-wells in 96-well plate frame
D07001	1x 96-well format Dog PXR assays
<b>Cynomolgus Monkey PXR Assay Kit Products</b>	
C07001-32	3x 32 Cyn Monkey PXR assays; strip-wells in 96-well plate frame
C07001	1x 96-well format Cyn Monkey PXR assays

<b>LIVE Cell Multiplex (LCM) Assay</b>	
LCM-01	Reagent volumes sufficient to perform <b>96</b> Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in <b>5x bulk volume</b> to perform <b>480</b> Live Cell Assays performed in 5 x 96-well assay plates
LCM-10	Reagent in <b>10x bulk volume</b> to perform <b>960</b> Live Cell Assays performed in 10 x 96-well assay plates
<b>INDIGlo Luciferase Detection Reagent</b>	
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.

[www.indigobiosciences.com](http://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.

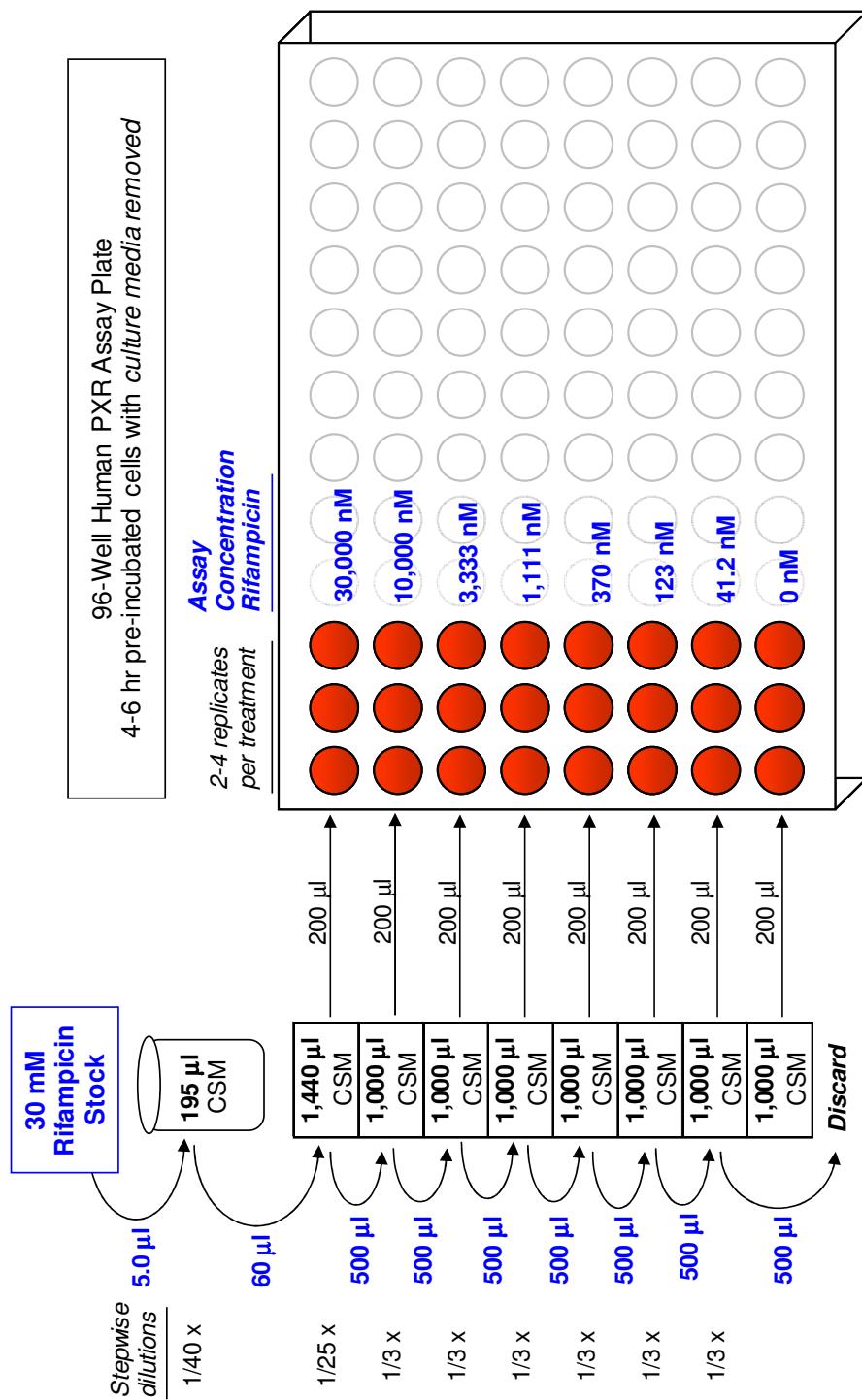
“CryoMite” is a Trademark <sup>TM</sup> of INDIGO Biosciences, Inc. (State College, PA, USA).

Product prices, availability, specifications, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most currently updated version.

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## APPENDIX 1

Example scheme for the serial dilution of Rifampicin reference agonist, and the setup of a Human PXR dose-response assay.





Nuclear Receptor & *In Vitro* Toxicology Solutions™

**Human Pregnane X Receptor  
(NR1I2, PXR, SXR)  
Reporter Assay System**

**3x 32 Assays in 96-well Format**  
Product # IB07001-32

■

**Technical Manual**  
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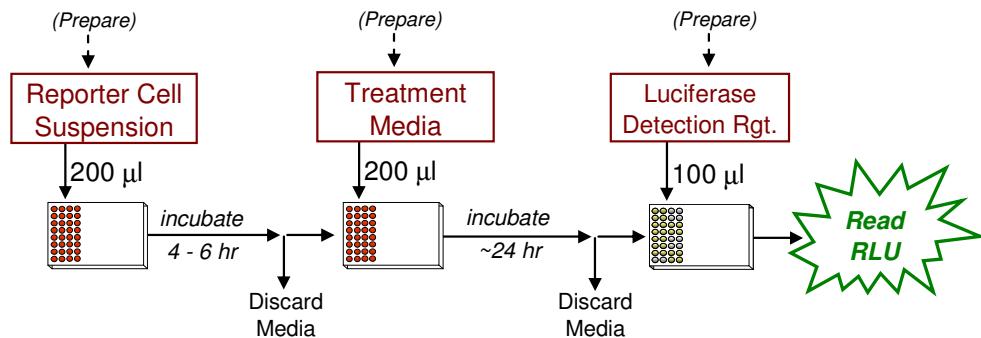
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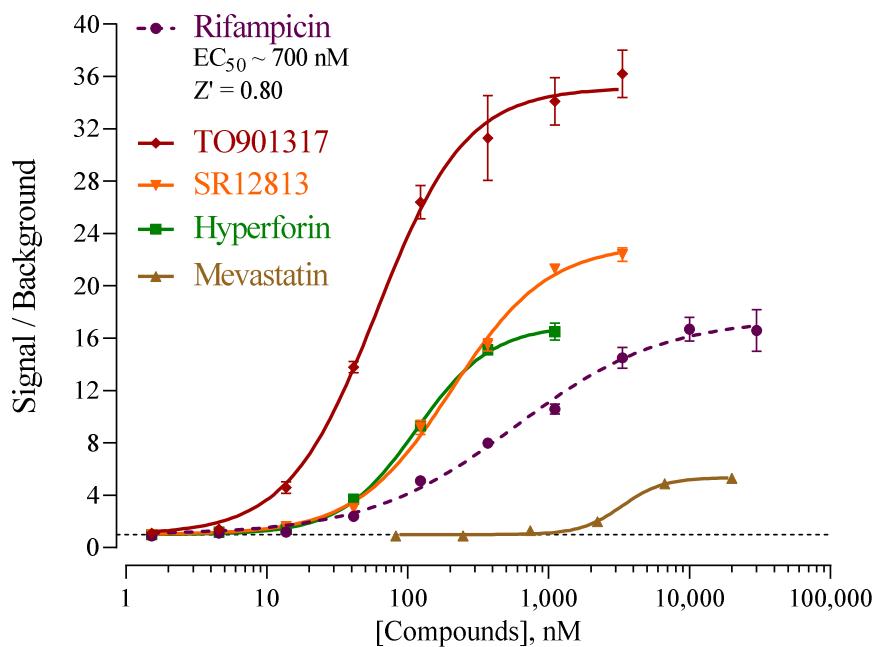
## ▪ Assay Scheme ▪

**Figure 1.** Assay workflow.

*In brief,* 200 µl of Reporter Cells is dispensed into wells of the assay plate and 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 hr incubation, discard the treatment media and add Luciferase Detection Reagent. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



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**Figure 2. Agonist dose-response analyses of Human PXR.**

Performance of the human PXR assay using the reference agonists Rifampicin (provided), Hyperforin dicyclohexylammonium (Enzo Life Sciences), TO901317(Cayman Chemical), SR12813 (Tocris), and Mevastatin (Cayman Chemical). Luminescence was quantified and average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n = 4). Fold-activation and Z' values were calculated as described by Zhang, *et al.* (1999)<sup>1</sup>. Non-linear regression and EC<sub>50</sub> analyses were performed using GraphPad Prism software. High Z' scores confirm the robust performance of this assay, and its suitability for HTS<sup>1</sup>.

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

## ***II. Product Components & Storage Conditions***

This Human PXR 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 inventory the individual kit components be sure to first transfer and submerge the tube of reporter cells in dry ice.

The aliquots of Reporter Cells are provided as single-use reagents. 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.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit.

<b><i>Kit Components</i></b>	<b><i>Amount</i></b>	<b><i>Storage Temp.</i></b>
▪ PXR Reporter Cells	3 x 0.6 mL	-80°C
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ Rifampicin, 30 mM (in DMSO) (reference agonist for PXR)	1 x 30 µL	-20°C
▪ Detection Substrate	3 x 2.0 mL	<b>-80°C</b>
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate frame	1	ambient
▪ Snap-in, 8-well strips (white, sterile, collagen-coated wells)	12	<b>-80°C</b>

*NOTE:* This Assay kit contains 8-well strips that have been collagen-coated and dried; these strip wells should be stored frozen (-20°C or colder) until use.

## ***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 (see *Step 2*)
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO<sub>2</sub> 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 deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- *Optional:* antagonist reference compound.
- *Optional:* clear 96-well assay plate, 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 1-11* are performed on **Day 1**, requiring less than 2 hours of actual bench work plus a 4 hr pre-incubation step. *Steps 12-17* 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<sub>50</sub> – EC<sub>85</sub>) of a known agonist AND varying concentrations of the test compound(s) to be evaluated for antagonist activity. This PXR Assay kit includes a 30 mM stock solution of **Rifampicin**, a low-potency agonist of PXR that may be used to setup antagonist-mode assays. 3  $\mu$ M Rifampicin typically approximates EC<sub>70-80</sub> in this cell-based assay. Hence, it presents a suitable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

Add the challenge agonist (rifampicin) to a bulk volume of **CSM** at an EC<sub>50</sub> – EC<sub>85</sub> concentration. This medium is then used to prepare serial dilutions of test compounds to achieve the desired respective final assay concentrations. We find that this is an efficient and precise method of setting up PXR antagonist assays, and it is the method presented in *Step 7b* of this protocol.

### 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 **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, or 3 tubes for 96 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 cell suspension will be **7.0 ml** per tube.

*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.

**3.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface with a 70% alcohol swab.**

**4.)** If more than one tube of Reporter cells was thawed, combine them and gently invert several times to gain a homogenous cell suspension. Dispense **200  $\mu$ l / well** of cell suspension into the mounted strip-wells.

*NOTE 4.1:* If INDIGO's Live Cell Multiplex Assay is to be incorporated then 'cell blank' wells (meaning cell-free, but containing 'CSM') must be included to allow quantification of plate-specific fluorescence background (refer to the LCMA Technical Manual).

*NOTE 4.2:* 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 4.3:* 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, preferably *collagen-coated*, 96-well assay plate. Continue to process this plate in identical manner to the white assay plate.

**5.) Pre-incubate reporter cells:** Place the assay plate into a mammalian cell incubator (37°C,  $\geq 70\%$  humidity, 5% CO<sub>2</sub>) for 4 - 6 hours.

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

**7.) 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 9*, the prepared treatment media will be dispensed at **200  $\mu$ l / well** into the strip-wells. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO carried over into assay reactions should not exceed 0.4%.

**a. Agonist-mode assays.** This Assay kit includes a 30 mM stock solution of Rifampicin, the most commonly cited reference agonist of human PXR. The following 7-point treatment series, prepared in serial 3-fold decrements, provides a complete dose-response: 30.0, 10.0, 3.33, 1.11, 0.370, 0.123 and 0.0412 $\mu$ M. Be sure to include 'no treatment', or 'vehicle only', control wells. **APPENDIX 1** 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 challenge agonist (rifampicin) to achieve the desired final assay-concentration (refer to "*A word about antagonist-mode assay setup*", pg. 7). The agonist-supplemented CSM is then used to generate dilutions of test compound stocks to achieve their final assay concentrations.

**8.)** At the end of the 4-6 hr pre-culture period, discard the pre-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 an 8-pin manifold (*e.g.*, Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus. Do *not* touch the well bottoms or run the tip of the aspiration device around the bottom circumference of the assay wells. Such practices will result in destruction of the cells and greatly increased well-to-well variability.

**9.)** Dispense **200  $\mu$ l / well** of each prepared treatment media into the assay plate.

**10.)** Transfer the assay plate into a cell culture incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity ( $\geq 70\%$ ) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

**11.)** 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.

**12.)** 30 minutes before intending to quantify receptor 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.

*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.

**13.)** 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*.

**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 8*).

**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
<b>Human PXR Assay Kit Products</b>	
IB07001-32	3x 32 Human PXR assays; strip-wells in 96-well plate frame
IB07001	1x 96-well format Human PXR assays
IB07002	1x 384-well format Human PXR assays
<b>Rat PXR Assay Kit Products</b>	
R07001-32	3x 32 Rat PXR assays; strip-wells in 96-well plate frame
R07001	1x 96-well format Rat PXR assays
<b>Mouse PXR Assay Kit Products</b>	
M07001-32	3x 32 Mouse PXR assays; strip-wells in 96-well plate frame
M07001	1x 96-well format Mouse PXR assays
<b>Dog PXR Assay Kit Products</b>	
D07001-32	3x 32 Dog PXR assays; strip-wells in 96-well plate frame
D07001	1x 96-well format Dog PXR assays
<b>Cynomolgus Monkey PXR Assay Kit Products</b>	
C07001-32	3x 32 Cyn Monkey PXR assays; strip-wells in 96-well plate frame
C07001	1x 96-well format Cyn Monkey PXR assays
<b>LIVE Cell Multiplex (LCM) Assay Products</b>	
LCM-01	Reagent volumes sufficient to perform <b>96</b> Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in <b>5x bulk volume</b> to perform <b>480</b> Live Cell Assays contained in 5 x 96-well assay plates
LCM-10	Reagent in <b>10x bulk volume</b> to perform <b>960</b> Live Cell Assays contained in 10 x 96-well assay plates

Please refer to INDIGO Biosciences website for updated product offerings.

[www.indigobiosciences.com](http://www.indigobiosciences.com)

## **VI. Limited Use Disclosures**

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

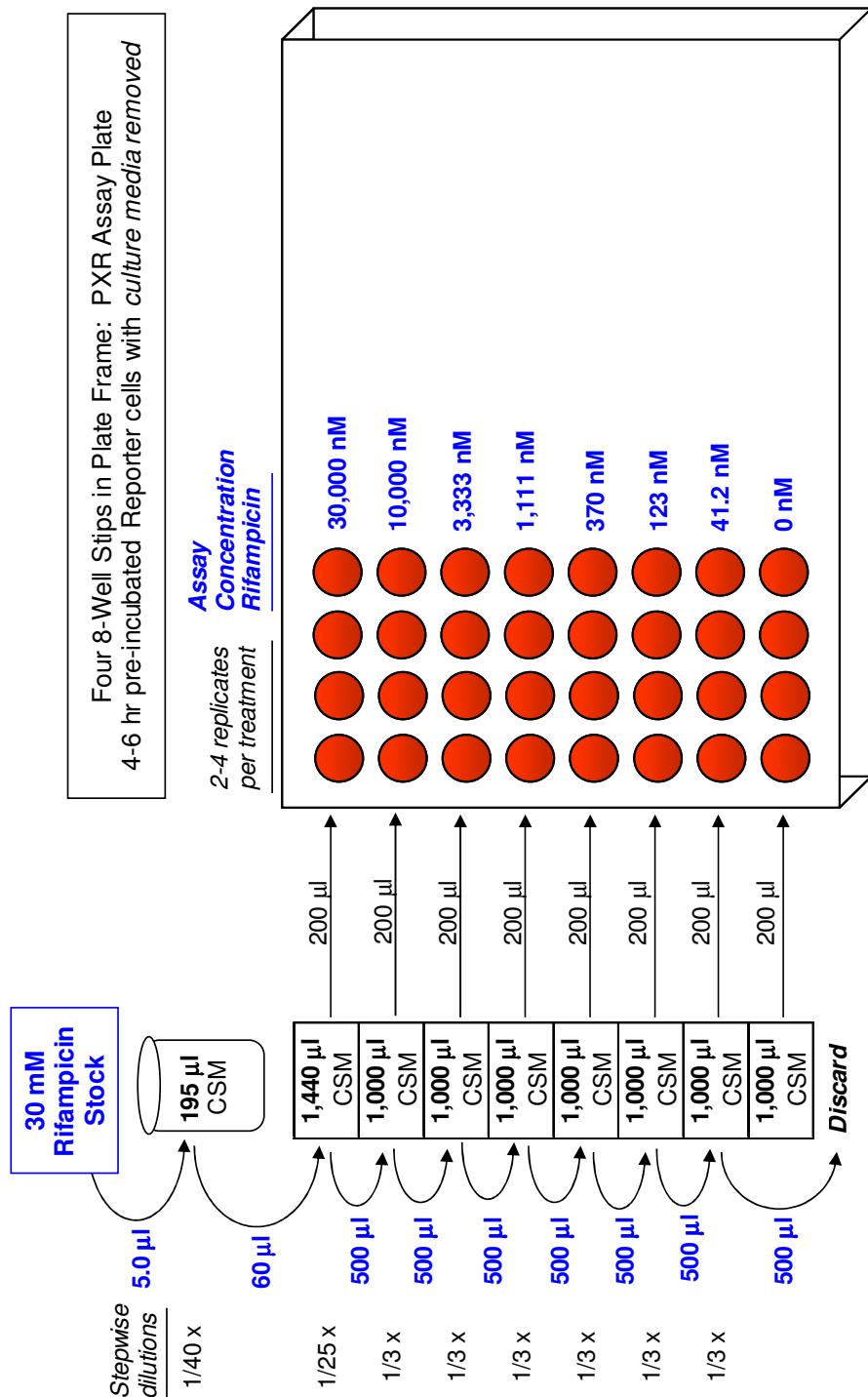
“CryoMite” is a Trademark™ of INDIGO Biosciences, Inc. (State College, PA, USA).

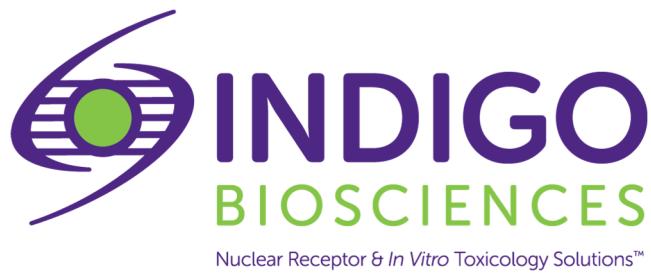
Product prices, availability, specifications, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most currently updated version.

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## APPENDIX 1

Example scheme for the serial dilution of Rifampicin reference agonist, and the setup of a Human PXR dose-response assay.





**Human Pregnane X Receptor  
(NR1I2, PXR, SXR)  
Reporter Assay System**

**384-well Format Assays**  
Product # IB07002

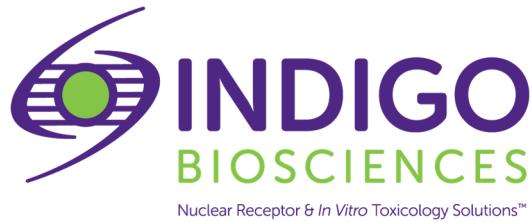
**Technical Manual**  
(*version 8.0*)

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## Human PXR Reporter Assay System 384-well Format Assays

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### APPENDIX 1a: Example Scheme for Serial Dilution when using tip-based dispensing of test compounds.....

### APPENDIX 1b: Example Scheme for Serial Dilutions when using acoustic dispensing of test compounds.....

## **I. Description**

### **▪ The Assay System ▪**

This assay product utilizes proprietary human cells engineered to provide constitutive, high-level expression of the **Human Pregnane X Receptor** (NR1I2), a ligand-dependent transcription factor commonly referred to as **PXR**. PXR is also known as the Steroid and Xenobiotic sensing nuclear receptor (**SXR**).

INDIGO's Reporter Cells express a hybrid form of human PXR. The N-terminal sequence encoding the PXR DNA binding domain (DBD) has been substituted with that of the yeast GAL4-DBD. The native PXR ligand binding domain (LBD) and other C-terminal domains remain intact and functional. Ligand interaction activates the receptor, causing it to bind to the GAL4 DNA binding sequence, which is functionally linked to a resident luciferase reporter gene. Thus, quantifying changes in luciferase activity in the treated reporter cells provides a sensitive surrogate measure of the changes in PXR activity. The principal application of this reporter assay kit is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against human PXR.

PXR 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 Assay kits provide the convenience of an all-inclusive cell-based assay system. In addition to PXR Reporter Cells, provided are two optimized media for use in recovering the cryopreserved cells and for diluting test samples. Also included is the reference agonist Rifampicin, Luciferase Detection Reagents, and a cell culture-ready assay plate.

### **▪ The Assay Chemistry ▪**

INDIGO's nuclear receptor reporter assays capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology.

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (*Photinus pyralis*). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg<sup>+2</sup>-dependent reaction that consumes O<sub>2</sub> and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP<sub>i</sub>, CO<sub>2</sub>, 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 Assays feature a luciferase detection reagent specially formulated to provide stable light emission between 30 and 100+ minutes after initiating the luciferase reaction. Incorporating a 30 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.

▪ **Considerations for the Preparation and Automated Dispensing of Test compounds ▪**

Small molecule compounds are typically solvated at high concentration (ideally 1,000x-concentrated) in DMSO and stored frozen as master stocks. For **384-well format assays** these master stocks will be diluted by one of two alternative methods, the selection of which will be dictated by the type of dispensing instrument that is to be used. This Technical Manual provides detailed protocols for each of these two alternative methods:

a.) Assay setups in which a conventional **tip-based** instrument is used to dispense test compounds into assay wells (in black text). Use **Compound Screening Medium (CSM)** to generate a series of **2x-concentration** test compound treatment media, as described in *Step 2a* of the **Assay Protocol**. The final concentration of DMSO carried over into assay reactions should never exceed 0.4%; strive to use 1,000x-concentrated stocks when they are prepared in DMSO.

*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.

*and,*

b.) Assay setups in which an **acoustic transfer** device is used to dispense test compounds into assay wells (text highlighted in blue). Use DMSO to make a series of **1,000x-concentrated** test compound stocks that correspond to each desired final assay concentrations, as described in *Step 2b* of the **Assay Protocol**.

▪ **Considerations for Automated Dispensing of Other Assay Reagents ▪**

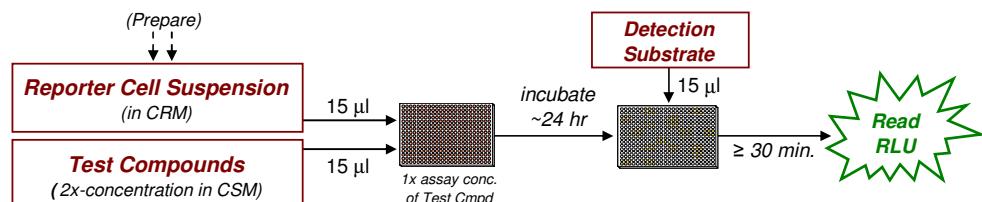
When dispensing into a small number of assay plates, first carefully consider the dead volume requirement of your tip-based dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses on a *per kit* basis. Always pool the individual reporter cell suspensions and all other respective assay kit reagents before processing multiple 384-well assay plates.

<b>Stock Reagent &amp; Volume provided</b>	<b>Volume to be Dispensed (384-well plate)</b>	<b>Excess rgt. volume available for instrument dead volume</b>
<i>when using tip dispensing of test cmpds</i> <b>Reporter Cell Suspension</b> 7.5 ml	15 µl / well 5.8 ml / plate	~ 1.7 ml
<i>when using acoustic dispensing of test cmpds</i> <b>Reporter Cell Suspension</b> 15 ml	30 µl / well 11.5 ml / plate	~ 3.4 ml
<b>Detection Substrate</b> 7.8 ml	15 µl / well 5.8 ml / plate	~ 2 ml

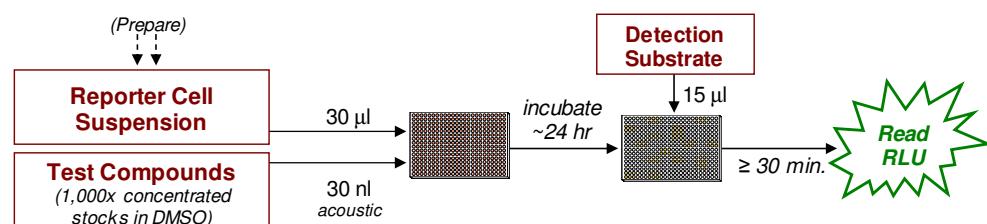
▪ Assay Scheme ▪

The Day 1 preparation, volumes, and chronology of dispensed cells and test compounds are different between assay setups using a *tip-based dispenser* (**1a**) and those using an *acoustic transfer device* (**1b**). Following 22 -24 hr incubation Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.

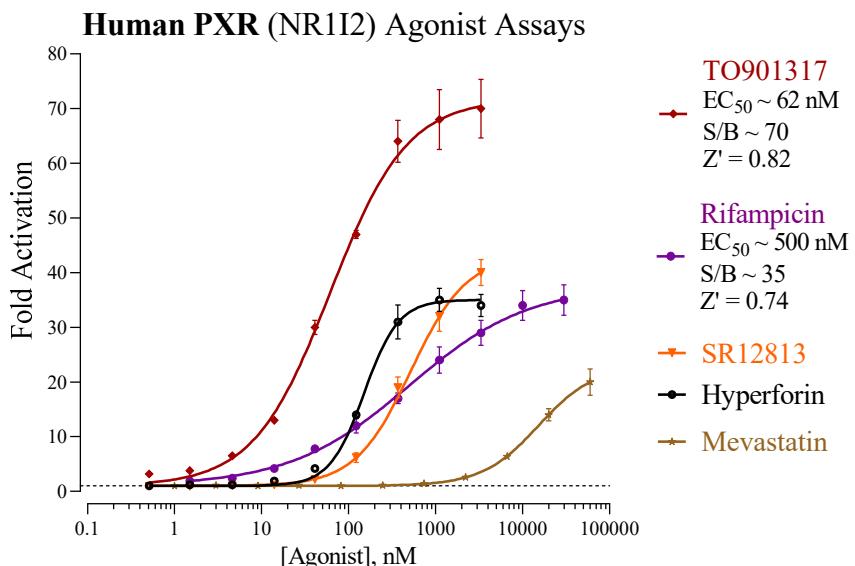
**Figure 1a.** Assay workflow if using conventional **tip-based** dispensing of test compounds.



**Figure 1b.** Assay workflow if using **acoustic** dispensing of test compounds.



▪ Assay Performance ▪



**Figure 2. Agonist dose-response of PXR.** PXR Reporter Cells were treated with the reference agonist Rifampicin (provided), TO901317, SR12813 and Mevastatin. Average Relative Light Units (RLU) and their respective values of Standard Deviation (SD), Coefficient of Variation (CV), and Fold-Activation (*i.e.*, S/B) were calculated for each treatment concentration ( $n = 4$ ).  $Z'$  was calculated as per Zhang, *et al.* (1999)<sup>1</sup>.

Treatment concentrations were Log10 transformed and respective RLU values were normalized as Fold-Activation. Data were plotted *via* non-linear regression and  $EC_{50}$  values were determined using GraphPad Prism software.

<sup>1</sup> 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 assay kit contains materials to perform assays in a single 384-well assay plate.

**Cryopreserved mammalian cells are temperature sensitive! To ensure maximal viability the tube of Reporter Cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in 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 inventory the individual kit components be sure to first transfer and submerge the tube of cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, the 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.

<b><i>Kit Components</i></b>	<b><i>Amount</i></b>	<b><i>Storage Temp.</i></b>
▪ PXR Reporter Cells	1 x 1.0 mL	<b>-80°C</b>
▪ Cell Recovery Medium (CRM)	1 x 7 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ Rifampicin, 30 mM (in DMSO) (reference agonist for PXR)	1 x 80 µL	-20°C
▪ Detection Substrate	1 x 7.8 mL	<b>-80°C</b>
▪ 384-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***

- dry ice container
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO<sub>2</sub> incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8-channel electronic, repeat-dispensing pipettes & tips suitable for dispensing 15 µL.
- disposable media basins, sterile.
- sterile multi-channel media basins *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 9-13* 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<sub>50</sub> – EC<sub>85</sub>) of a known agonist AND varying concentrations of the test compound(s) to be evaluated for antagonist activity. This assay kit includes a 30 mM stock solution of **Rifampicin**, a low-potency but commonly used reference agonist of PXR that may be used to setup antagonist-mode assays. 5  $\mu$ M Rifampicin typically approximates EC<sub>70-80</sub> in this assay. Hence, it presents a reasonable *final 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 protocol when performing tip-based dispensing, and *Step 6b of the protocol when using an acoustic transfer device to dispense test compounds*.

Note that when using a *tip-based instrument* for the dispensing of 2x-concentrated test compounds the cell suspension must also be supplemented with a **2x**-concentration of the challenge agonist.

**When using an acoustic transfer device for the dispensing of 1,000x-concentrated test compounds the cell suspension should be supplemented with a **1x**-concentration of the challenge 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. NOTE that test and reference compounds will be prepared differently when using tip-dispensing *vs.* **acoustic dispensing**. Regardless of the method, the total DMSO carried over into assay reactions should never exceed 0.4%.

- a. *Tip dispensing method:* In *Step 6*, 15  $\mu$ l / well of the prepared treatment media is added to the assay that has been pre-dispensed with 15  $\mu$ l /well 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. Plan dilution volumes carefully; this assay kit provides 35 ml of CSM.
- b. *Acoustic dispensing method:* In *Step 6*, 30 nl / well of **1,000x**-concentrated test compound solutions (prepared in DMSO) are added to the assay plate using an acoustic transfer device.

**Preparing the positive control:** This assay kit includes a 30 mM stock solution of **Rifampicin**, a commonly used reference agonist of PXR that may be used to setup antagonist-mode assays. The following 7-point treatment series, with concentrations presented in 3-fold decrements, provides a complete dose-response: 30, 10, 3.33, 1.11, 0.370, 0.123, and 0.0412  $\mu$ M. Always include a 'no treatment' (or 'vehicle') control.

**APPENDIX 1a** provides an example for generating such a dilution series to be used when *tip-dispensing* compound solutions prepared in CSM (15  $\mu$ l / well).

**APPENDIX 1b** provides an example for generating such a series of 1,000x-concentrated solutions of compounds prepared in DMSO to be used when performing *acoustic dispensing* (30 nl / well).

### When using **tip-based** instrumentation for dispensing test compounds ...

3.) *First*, retrieve the tube of **CRM** from the 37°C water bath, sanitize the outside with a 70% ethanol swab;

*Second*, retrieve **Reporter Cells** from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. Perform a *rapid thaw* of the frozen cells by transferring a **6.5 ml** volume of 37°C CRM into the tube of frozen cells. Recap the tube of Reporter Cells and place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.5 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, then transfer it into the cell culture hood.

5.) Gently invert the tube of cell suspension several times to disperse cell aggregates and gain a homogenous suspension.

a. **for Agonist-mode assays:** Dispense **15 µl / well** of cell suspension into the Assay Plate.

~ or ~

b. **for Antagonist-mode assays:** Supplement the bulk volume of Reporter Cells suspension with a 2x-concentration of the challenge agonist (refer to "A word about antagonist-mode assay setup", pg. 7). Dispense **15 µl / well** of cell suspension into the Assay Plate.

6.) Dispense **15 µl / well** of 2x-concentrated treatment media (from *Step 2a*) into the assay plate.

### When using an **acoustic transfer** device for dispensing test compounds ...

3.) Dispense **30 nl / well** of the 1,000x-concentrated compounds (in DMSO solutions, from *Step 2b*) into the assay plate.

4.) *First*, retrieve the tube of **CRM** from the 37°C water bath, sanitize the outside with a 70% ethanol swab;

*Second*, retrieve **Reporter Cells** from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. Perform a *rapid thaw* of the frozen cells by transferring a **6.5 ml** volume of 37°C CRM into the tube of frozen cells. Recap the tube of cells and place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.5 ml.

5.) Retrieve the tube of cell suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab. Add an additional **7.5 ml** of **CSM** to the tube. The resulting volume of cell suspension will be 15 ml.

6.) Gently invert the tube of cells several times to disperse cell aggregates and gain a homogenous cell suspension.

a. **for Agonist-mode assays:** Dispense **30 µl / well** of cell suspension into the Assay Plate that has been pre-dispensed with test compounds.

~ or ~

b. **for Antagonist-mode assays:** Supplement a bulk volume of CSM with the challenge agonist Rifampicin to achieve an EC<sub>50</sub> – EC<sub>80</sub> concentration (refer to "A word about antagonist-mode assay setup", pg. 7). Dispense **30 µl / well** of cell suspension into the Assay Plate that has been pre-dispensed with test compounds.

**NOTE:** 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.

*(continued ...)*

*NOTE:* Following the dispensing of Reporter Cells and test compounds INDIGO recommends performing a *low-speed* spin of the assay plate (with lid) for 1-2 minutes using a room temperature centrifuge fitted with counter-balanced plate carriers.

- 7.) Transfer the assay plate into a 37°C, humidified, 5% CO<sub>2</sub> incubator for 22 - 24 hours.

*NOTE:* Ensure a high-humidity ( $\geq 85\%$ ) 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** from freezer storage and place 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.) Approximately 30 minutes before intending to quantify receptor activity remove **Detection Substrate** from the refrigerator and place it in a low-light area so that it may equilibrate to room temperature. Gently invert the tube several times to ensure a homogenous solution.

*NOTE:* Do NOT actively warm Detection Substrate above room temperature. If this solution was 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.) Following 22 - 24 hours of incubation dispense **15  $\mu$ l / well** of **Detection Substrate** to the assay plate.

*NOTE: Perform this reagent transfer 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 will degrade the accuracy and precision of the assay data. INDIGO recommends performing a final *low-speed* spin of the assay plate (with lid) for 1-2 minutes using a room temperature centrifuge fitted with counter-balanced plate carriers.

- 12.) Allow the plate(s) to rest at room temperature for 30 minutes. Do not shake the assay plate(s) during this period.

*NOTE:* the luminescent signal is unstable during the first 30 minutes of the luciferase reaction, however, after the initial 30 minute reaction period the luminescence signal achieves a stable emission output.

- 13.) Quantify luminescence.

## ***V. Related Products***

<b>Human PXR Assay Kit Products</b>	
<b>Product No.</b>	<b>Product Descriptions</b>
IB07001-32	3x 32 Human PXR assays; 8-well strips (96-well plate format)
IB07001	1x 96-well format Human PXR assays
IB07002	1x 384-well format Human PXR assays
<b>Rat PXR Assay Kit Products</b>	
R07001-32	3x 32 Rat PXR assays; 8-well strips (96-well plate format)
R07001	1x 96-well format Rat PXR assays
<b>Mouse PXR Assay Kit Products</b>	
M07001-32	3x 32 Mouse PXR assays; 8-well strips (96-well plate format)
M07001	1x 96-well format Mouse PXR assays
<b>Dog PXR Assay Kit Products</b>	
D07001-32	3x 32 Dog PXR assays; 8-well strips (96-well plate format)
D07001	1x 96-well format Dog PXR assays
<b>Cynomolgus Monkey PXR Assay Kit Products</b>	
C07001-32	3x 32 Cynomolgus Monkey PXR assays; 8-well strips (96-well plate format)
C07001	1x 96-well format Cynomolgus Monkey PXR assays

Please refer to INDIGO Biosciences website for updated product offerings.

**[www.indigobiosciences.com](http://www.indigobiosciences.com)**

## ***VI. Limited Use Disclosures***

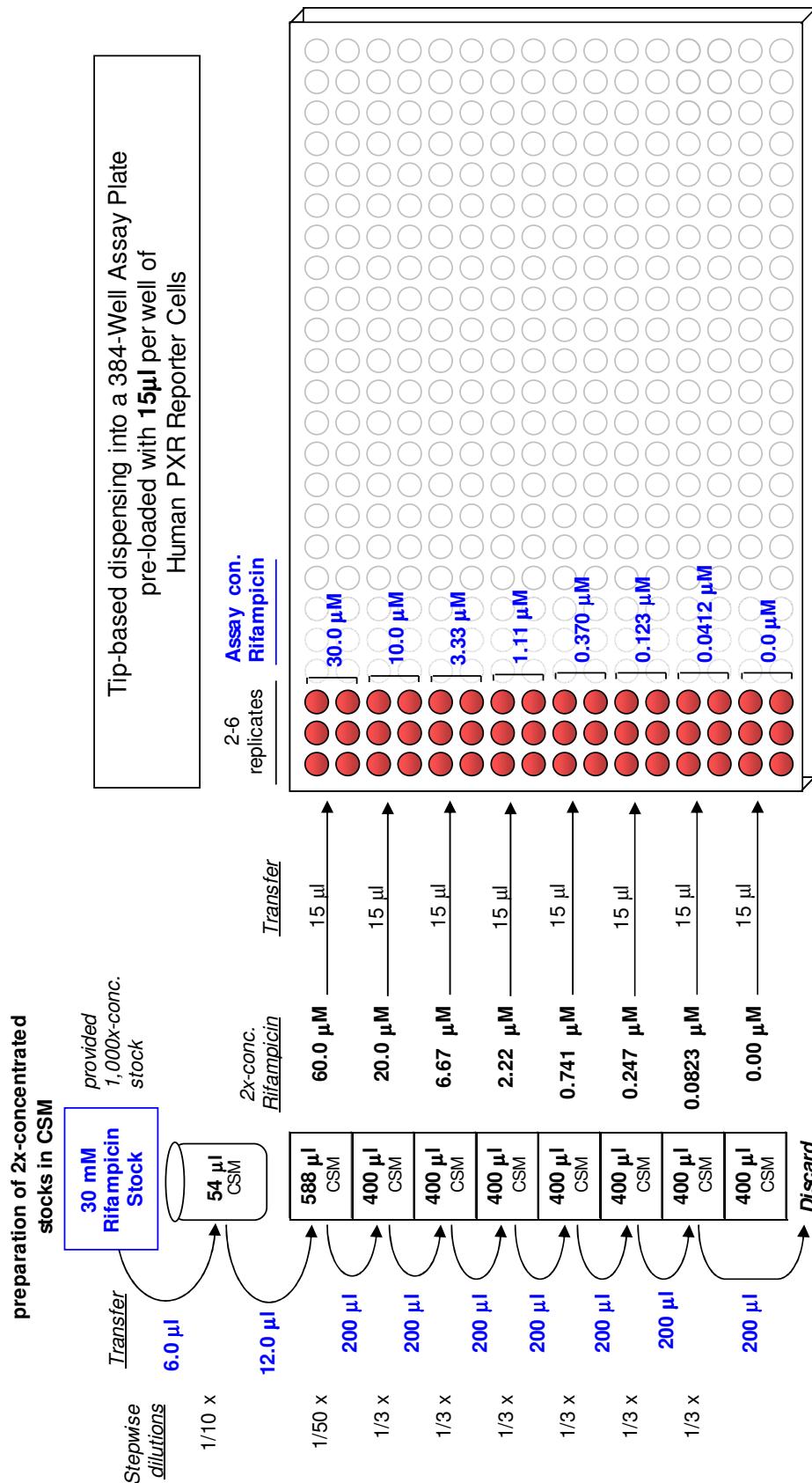
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**APPENDIX 1a for tip-based dispensing.** Example scheme for the serial dilution of the reference agonist Rifampicin into CSM to generate **2x-concentrated** treatment media. A *tip-based* instrument is used to dispense 15  $\mu$ l / well into an assay plate that has been *pre-dispensed* with 15  $\mu$ l / well of PXR Reporter Cells suspension.



**APPENDIX 1b for acoustic dispensing.** Example scheme for the serial dilution of the reference agonist Rifampicin into DMSO to generate **1,000x-concentrated stocks**. 30 nl / well are pre-dispensed into an empty assay plate using an acoustic transfer device.

