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

96-well Format Assays
Product # IB07001

Technical Manual
(version 7.1)

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Human PXR Reporter Assay System
96-well Format Assays

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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 binds 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 principle application of this reporter assay system is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against 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 Bioscience’s Nuclear Receptor Reporter 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, a reference agonist, Luciferase Detection Reagent, and a cell culture-ready assay plate.

- The Assay Chemistry -

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

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (Photinus pyralis). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg$^{2+}$-dependent reaction that consumes O$_2$ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP$_i$, CO$_2$, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer, and is reported in terms of Relative Light Units (RLU’s).

INDIGO’s Nuclear Receptor Assay Systems feature a luciferase detection reagent specially formulated to provide stable light emission between 5 and 90+ minutes after initiating the luciferase reaction. Incorporating a 5 minute reaction-rest period ensures that light emission profiles attain maximal stability, thereby allowing assay plates to be processed in batch. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.
**Preparation of Test Compounds**

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using **Compound Screening Medium (CSM)**, as described in Step 2 of the **Assay Protocol**. This method avoids the adverse effects of introducing high concentrations of DMSO into the assay. The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

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

**Considerations for Automated Dispensing**

When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, “dead volume” is the volume of reagent that is dedicated to the instrument; it will **not** be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

<table>
<thead>
<tr>
<th>Stock Reagent &amp; Volume provided</th>
<th>Volume to be Dispensed (96-well plate)</th>
<th>Excess rgt. volume available for instrument dead volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reporter Cell Suspension 12 ml <em>(prepared from kit components)</em></td>
<td>100 µl / well 9.6 ml / plate</td>
<td>~ 2.4 ml</td>
</tr>
<tr>
<td>LDR 12 ml <em>(prepared from kit components)</em></td>
<td>100 µl / well 9.6 ml / plate</td>
<td>~ 2.4 ml</td>
</tr>
</tbody>
</table>

**Assay Scheme**

**Figure 1.** Assay workflow. **In brief,** Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user’s test compounds. Following 22-24 hr incubation, treatment media are discarded and prepared Luciferase Detection Reagent (LDR) is added. Light emission from each assay well is quantified using a plate-reading luminometer.
Assay Performance

Human PXR (NR1I2): Agonist assays

![Graph](https://via.placeholder.com/150)

- **Rifampicin**
  - EC$_{50}$ = 2.5 µM
  - Hill slope = 1.17
  - S/B = 15;
  - Z' = 0.84

- TO901317

- SR12813

- Hyperforin dicyclohexylammonium

- Troglitazone

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 Troglitazone (Cayman Chemical). Luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 6). Fold-activation and Z’ values were calculated as described by Zhang, et al. (1999). Non-linear regression and EC$_{50}$ analyses were performed using GraphPad Prism software. High Z’ scores confirm the robust performance of this assay, and it's suitability for HTS.$^1$


$$Z' = 1 - \frac{3*(SD_{Control} + SD_{Background})}{(RLU_{Control} - RLU_{Background})}$$
II. Product Components & Storage Conditions

This Human PXR Reporter Assay System contains materials to perform assays in a single collagen-coated 96-well assay plate.

The aliquot of PXR Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

Assay kits are shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels. Alternatively, the entire kit may be further stored at -80°C.

To ensure maximal viability, “Reporter Cells” must be maintained at -80°C until immediately prior to use.

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

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>• PXR Reporter Cells</td>
<td>1 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Cell Recovery Medium (CRM)</td>
<td>1 x 10.5 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Compound Screening Medium (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Rifampicin, 30 mM (in DMSO)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>(reference agonist for PXR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Detection Substrate</td>
<td>1 x 6.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Detection Buffer</td>
<td>1 x 6.0 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• 96-well, collagen-coated assay plate</td>
<td></td>
<td>-20°C</td>
</tr>
<tr>
<td>(white, sterile, cell-culture ready)</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

*NOTE:* This PXR Assay System 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**
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8- or 12-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media basins, sterile.
- sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), or deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- antagonist reference compound (optional).

**DAY 2** plate-reading luminometer.
**IV. Assay Protocol**

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-8* are performed on **Day 1**, requiring less than 2 hours to complete. *Steps 9-15* are performed on **Day 2**, and require less than 1 hour to complete.

- **A word about Antagonist-mode assay setup**

Receptor inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between $EC_{50} – EC_{85}$) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This PXR Reporter Assay System kit includes a 30 mM stock solution of **Rifampicin**, a low-potency agonist of PXR that may be used to setup antagonist-mode assays. 5 µM Rifampicin typically approximates $EC_{80}$ in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

We find that adding the reference agonist to the bulk suspension of Reporter Cells (i.e., prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in **Step 5b** of the following protocol. Note that, in **Step 6**, 100 µl of treatment media is combined with 100 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.

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

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

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

   The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

   Note that, in **Step 6**, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl of Reporter Cells. Hence, to achieve the desired *final* assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Use **CSM** to prepare the appropriate dilution series. Manage dilution volumes carefully. This assay kit provides 35 ml of CSM.

   **Preparing the positive control:** This PXR Reporter Assay System kit includes a 30 mM stock solution of **Rifampicin**, a commonly cited (but low-potency) reference agonist of human PXR. We find that the following 7-point treatment series, prepared in serial 3-fold decrements, provides a suitable dose-response: 30.0, 10.0, 3.33, 1.11, 0.370, 0.123 and 0.0412 µM (final assay concentrations), and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

3.) **Rapid Thaw of the Reporter Cells:** *First*, retrieve the tube of **CRM** from the 37°C water bath and sanitize the outside surface with a 70% ethanol swab.

   *Second*, retrieve **Reporter Cells** from -80°C storage and, *without delay*, perform a rapid thaw of the frozen cells by transferring a 10 ml volume of the pre-warmed CRM into the tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 12 ml.

4.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface of the tube with a 70% alcohol swab.
5.) a. Agonist-mode assays. Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 \( \mu l \) of cell suspension into each well of the Assay Plate.

   - or -

b. Antagonist-mode assays. Gently invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain an homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "A word about antagonist-mode assay setup", pg. 7). Dispense 100 \( \mu l \) of cell suspension into each well of the Assay Plate.

   NOTE 5.1: Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate will cause well-to-well variation (= increased Standard Deviation) in the assay.

   NOTE 5.2: Users sometimes prefer to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear 96-well collagen-coated plate, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense 100 \( \mu l \) of 2x-concentration treatment media into appropriate assay wells.

7.) Transfer the assay plate into a 37°C, humidified 5% CO\(_2\) incubator for 22 - 24 hours.

   NOTE: Ensure a high-humidity (\( \geq 85\%) \) 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 and Detection Buffer from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.

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

9.) 30 minutes before intending to quantify PXR activity, remove Detection Substrate and Detection Buffer from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature. Once at room temperature, gently invert each tube several times to ensure homogenous solutions.

   NOTE: Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, use a room temperature water bath to expedite thawing.

10.) Set the plate-reader to "luminescence" mode. Set the instrument to perform a single 5 second “plate shake” prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, or less.

11.) Immediately before proceeding to Step 12, transfer the entire volume of Detection Buffer into the vial of Detection Substrate, thereby generating a 12 ml volume of Luciferase Detection Reagent (LDR). Mix gently to avoid foaming.

12.) Following 22 - 24 hours of incubation discard all media contents by ejecting it into an appropriate waste container. Gently tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

13.) Add 100 \( \mu l \) of LDR to each well of the assay plate. Allow the assay plate to rest at room temperature for at least 5 minutes. Do not shake the assay plate during this period.

14.) Quantify luminescence.
### V. Related Products

#### Human PXR Assay Kit Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB07001-32</td>
<td>3x 32 Human PXR assays; strip-wells in 96-well plate frame</td>
</tr>
<tr>
<td>IB07001</td>
<td>1x 96-well format Human PXR assays</td>
</tr>
<tr>
<td>IB07002</td>
<td>1x 384-well format Human PXR assays</td>
</tr>
</tbody>
</table>

Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

#### Rat PXR Assay Kit Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
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<tbody>
<tr>
<td>R07001-32</td>
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#### LIVE Cell Multiplex (LCM) Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
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<tbody>
<tr>
<td>LCM-01</td>
<td>Reagents to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-05</td>
<td>Reagents in 5x-bulk volume to perform 480 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagent in 10x-bulk volume to perform 960 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
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Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

### VI. Limited Use Disclosures

The method of recombinant expression of the steroid and xenobiotic sensing nuclear receptor (SXR) is the subject of U.S. Patent 6,756,491. INDIGO Biosciences, Inc. has entered into a sub-license agreement with Puracyp, Inc. (Carlsbad, CA) conferring the right to utilize SXR / PXR sequences to independently develop assay kit products and services.

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

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

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

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APPENDIX 1

Example scheme for the serial dilution of Rifampicin reference agonist, and the setup of a PXR dose-response assay.
Human Pregnane X Receptor
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Reporter Assay System

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

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**Assay Scheme**

*Figure 1.* Assay workflow. *In brief,* Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user’s test compounds. Following 22-24 hr incubation, treatment media are discarded and prepared Luciferase Detection Reagent (LDR) is added. Light emission from each sample well is quantified using a plate-reading luminometer.
***Assay Performance***

**Human PXR (NR1I2): Agonist assays**

![Figure 2. Agonist dose-response analyses of Human PXR.](image)

Performance of the human PXR assay using the reference agonists Rifampicin (provided), Hyperforin dicyclohexylammonium (Enzo Life Sciences), TO901317 (Cayman Chemical), SR12813 (Tocris), and Troglitazone (Cayman Chemical). Luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 6). Fold-activation and Z’ values were calculated as described by Zhang, *et al.* (1999). Non-linear regression and EC₅₀ analyses were performed using GraphPad Prism software. High Z’ scores confirm the robust performance of this assay, and it's suitability for HTS.


\[
Z' = 1 - \frac{3 \times (SD_{\text{Control}} + SD_{\text{Background}})}{(RLU_{\text{Control}} - RLU_{\text{Background}})}
\]
II. Product Components & Storage Conditions

This Human PXR Reporter Assay System contains materials to perform three distinct
groups of assays in a 96-well plate format. 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.

The individual 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.

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
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<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
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<td>• PXR Reporter Cells</td>
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<td>-80°C</td>
</tr>
<tr>
<td>• Cell Recovery Medium (CRM)</td>
<td>1 x 10.5 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Compound Screening Medium (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Rifampicin, 30 mM (in DMSO)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>(reference agonist for PXR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Detection Substrate</td>
<td>3 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Detection Buffer</td>
<td>3 x 2.0 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Plate frame</td>
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<td>ambient</td>
</tr>
<tr>
<td>• Snap-in, 8-well strips</td>
<td>12</td>
<td>ambient</td>
</tr>
<tr>
<td>(white, sterile, cell-culture ready)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE: This PXR Assay System 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
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• 37°C water bath.
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• disposable media basins, sterile.
• sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function
  Solution Basin"), or deep-well plates, or appropriate similar vessel for generating dilution
  series of reference compound(s) and test compound(s).
• antagonist reference compound (optional).

DAY 2 plate-reading luminometer.
**IV. Assay Protocol**

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. Steps 1-8 are performed on **Day 1**, requiring less than 2 hours to complete. Steps 9-15 are performed on **Day 2**, and require less than 1 hour to complete.

- **A word about Antagonist-mode assay setup**

Receptor inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between EC$_{50}$ – EC$_{85}$) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This PXR Reporter Assay System kit includes a 30 mM stock solution of **Rifampicin**, a low-potency agonist of PXR that may be used to setup antagonist-mode assays. 5 µM Rifampicin typically approximates EC$_{80}$ in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

We find that adding the reference agonist to the bulk suspension of Reporter Cells (i.e., prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in Step 5b of the following protocol. Note that, in Step 6, 100 µl of treatment media is combined with 100 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.

---

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

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

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

   The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

   Note that, in Step 6, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl of Reporter Cells. Hence, to achieve the desired final assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Use **CSM** to prepare the appropriate dilution series. Manage dilution volumes carefully. This assay kit provides 35 ml of CSM.

   **Preparing the positive control:** This PXR Reporter Assay System kit includes a 30 mM stock solution of **Rifampicin**, a commonly cited (but low-potency) reference agonist of human PXR. We find that the following 7-point treatment series, prepared in serial 3-fold decrements, provides a suitable dose-response: 30.0, 10.0, 3.33, 1.11, 0.370, 0.123 and 0.0412 µM (final assay concentrations), and including a ‘no treatment’ control. **APPENDIX 1** provides an example for generating such a dilution series.

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

   Second, retrieve **Reporter Cells** from -80°C storage: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, and 3 tubes for 96 assay wells. Without delay, Perform a rapid thaw of the frozen cells by transferring a 3.0 ml volume of 37°C CRM into each tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. If only one tube of reporter cells is thawed (32 assays), the resulting volume of cell suspension will be 3.6 ml.

   Third, work in the cell culture hood to carefully mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.
4.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface of the tube with a 70% alcohol swab.

5.) *a. Agonist-mode assays.* Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into each well of the Assay Plate.

   ~ or ~

*b. Antagonist-mode assays.* Gently invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain an homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to “A word about antagonist-mode assay setup”, pg. 7). Dispense 100 µl of cell suspension into each well of the Assay Plate.

   NOTE 5.1: Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate will cause well-to-well variation (= increased Standard Deviation) in the assay.

   NOTE 5.2: Users sometimes prefer to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear assay plate, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense 100 µl of 2x-concentration treatment media into appropriate assay wells.

7.) Transfer the assay plate into a 37°C, humidified 5% CO$_2$ incubator for 22 - 24 hours.

   NOTE: Ensure a high-humidity (≥ 85%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious “edge-effects” in the assay plate.

8.) For greater convenience on Day 2, retrieve Detection Substrate and Detection Buffer from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.

9.) 30 minutes before intending to quantify PXR activity, remove Detection Substrate and Detection Buffer from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature. Once at room temperature, gently invert each tube several times to ensure homogenous solutions.

   NOTE: Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, use a room temperature water bath to expedite thawing.

10.) Set the plate-reader to “luminescence” mode. Set the instrument to perform a single 5 second “plate shake” prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, or less.

11.) *Immediately before proceeding to Step 12:* 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.

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

   NOTE: Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media via a sweeping downward movement 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.

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

14.) Quantify luminescence.
V. Related Products

### Human PXR Assay Kit Products

<table>
<thead>
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<tr>
<td>IB07002</td>
<td>1x 384-well format Human PXR assays</td>
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Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

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<td>Reagents in 5x-bulk volume to perform 480 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagent in 10x-bulk volume to perform 960 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
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Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Limited Use Disclosures

The method of recombinant expression of the steroid and xenobiotic sensing nuclear receptor (SXR) is the subject of U.S. Patent 6,756,491. INDIGO Biosciences, Inc. has entered into a sub-license agreement with Puracyp, Inc. (Carlsbad, CA) conferring the right to utilize SXR / PXR sequences to independently develop assay kit products and services.

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

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

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

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APPENDIX 1

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

APPENDIX 1

Example scheme for the serial dilution of Rifampicin reference agonist, and the setup of a PXR dose-response assay.
Human Pregnane X Receptor
(NR1I2, PXR, SXR)
Reporter Assay System

384-well Format Assays
Product # IB07002

Technical Manual
(version 7.1)

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Human PXR Reporter Assay System
384-well Format Assays

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• Preparation of Test Compounds ............................................ 4
• Considerations for Automated Dispensing .............................. 4
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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 principle application of this reporter assay system is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against 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 Bioscience’s Nuclear Receptor Reporter 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, a reference agonist, Luciferase Detection Reagent, and a cell culture-ready assay plate.

- **The Assay Chemistry**

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

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (Photinus pyralis). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg\(^{2+}\)-dependent reaction that consumes O\(_2\) and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP\(_i\), CO\(_2\), and photon emission. Luminescence intensity of the reaction is quantified using a luminometer, and is reported in terms of Relative Light Units (RLU’s).

INDIGO’s Nuclear Receptor Assay Systems 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.
**Preparation of Test Compounds**

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using Compound Screening Medium (CSM), as described in Step 2 of the Assay Protocol. This method avoids the adverse effects of introducing high concentrations of DMSO into the assay. The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

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

**Considerations for Automated Dispensing**

When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, “dead volume” is the volume of reagent that is dedicated to the instrument; it will not be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

<table>
<thead>
<tr>
<th>Stock Reagent &amp; Volume provided</th>
<th>Volume to be Dispensed (384-well plate)</th>
<th>Excess rgt. volume available for instrument dead volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reporter Cell Suspension 7.5 ml (prepared from kit components)</td>
<td>15 µl / well 5.8 ml / plate</td>
<td>~ 1.7 ml</td>
</tr>
<tr>
<td>Detection Substrate 7.8 ml</td>
<td>15 µl / well 5.8 ml / plate</td>
<td>~ 2 ml</td>
</tr>
</tbody>
</table>

**Assay Scheme**

*Figure 1.* Assay workflow. *In brief,* the prepared suspension of thawed Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user’s test compounds. Following 22–24 hr incubation Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.
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 Troglitazone (Cayman Chemical). Luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 6). Fold-activation and Z’ values were calculated as described by Zhang, et al. (1999). Non-linear regression and EC_{50} analyses were performed using GraphPad Prism software. High Z’ scores confirm the robust performance of this assay, and it’s suitability for HTS.

Z’ = 1 - \{3*(SD^Control + SD^Background) / (RLU^Control - RLU^Background)}\]
II. Product Components & Storage Conditions

This Human PXR Reporter Assay System contains materials to perform assays in a single collagen-coated 384-well assay plate.

The aliquot of PXR Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

Assay kits are shipped on dry ice. Upon receipt, individual kit components may be stored at the temperatures indicated on their respective labels. Alternatively, the entire kit may be further stored at -80°C.

To ensure maximal viability, “Reporter Cells” must be maintained at -80°C until immediately prior to use.

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

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>• PXR Reporter Cells</td>
<td>1 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Cell Recovery Medium (CRM)</td>
<td>1 x 6 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Compound Screening Medium (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Rifampicin, 30 mM (in DMSO) (reference agonist for PXR)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Detection Substrate</td>
<td>1 x 7.8 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• 384-well, collagen-coated assay plate (white, sterile, cell-culture ready)</td>
<td>1</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

**NOTE:** This PXR Assay System contains one 384-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**
• cell culture-rated laminar flow hood.
• 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
• 37°C water bath.
• 70% alcohol wipes
• 8- or 12-channel electronic, repeat-dispensing pipettes & sterile tips
• disposable media basins, sterile.
• sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), or deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
• antagonist reference compound (optional).

**DAY 2** plate-reading luminometer.
IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. Steps 1-8 are performed on Day 1, requiring less than 2 hours to complete. Steps 9-15 are performed on Day 2, and require less than 1 hour to complete.

- A word about Antagonist-mode assay setup -

Receptor inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between EC$_{50}$ – EC$_{85}$) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This PXR Reporter Assay System kit includes a 30 mM stock solution of Rifampicin, a low-potency agonist of PXR that may be used to setup antagonist-mode assays. 5 µM Rifampicin typically approximates EC$_{80}$ in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

We find that adding the reference agonist to the bulk suspension of Reporter Cells (i.e., prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in Step 5b of the following protocol. Note that, in Step 6, 15 µl of treatment media is combined with 15 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. APPENDIX 1 provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.

---

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

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

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

   The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

   Note that, in Step 6, 15 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 15 µl of Reporter Cells. Hence, to achieve the desired final assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Use CSM to prepare the appropriate dilution series. Manage dilution volumes carefully. This assay kit provides 35 ml of CSM.

   **Preparing the positive control:** This PXR Reporter Assay System kit includes a 30 mM stock solution of Rifampicin, a commonly cited (but low-potency) reference agonist of human PXR. We find that the following 7-point treatment series, prepared in serial 3-fold decrements, provides a suitable dose-response: 30.0, 10.0, 3.33, 1.11, 0.370, 0.123 and 0.0412 µM (final assay concentrations), and including a 'no treatment' control. APPENDIX 1 provides an example for generating such a dilution series.

3.) Rapid Thaw of the Reporter Cells: First, retrieve the tube of CRM from the 37°C water bath and sanitize the outside surface with a 70% ethanol swab.

   Second, retrieve Reporter Cells from -80°C storage and, without delay, perform a rapid thaw of the frozen cells by transferring a 5.5 ml volume of the pre-warmed CRM into the tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.5 ml.

4.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface of the tube with a 70% alcohol swab.
5.) a. **Agonist-mode assays.** Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 15 µl of cell suspension into each well of the Assay Plate.

b. **Antagonist-mode assays.** Gently invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain an homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "A word about antagonist-mode assay setup", pg. 7). Dispense 15 µl of cell suspension into each well of the Assay Plate.

**NOTE 5.1:** Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate will cause well-to-well variation (= increased Standard Deviation) in the assay.

**NOTE 5.2:** Users sometimes prefer to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear 384-well collagen-coated assay plate, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense 15 µl of 2x-concentration treatment media into appropriate assay wells.

7.) Transfer the assay plate into a 37°C, humidified 5% CO₂ incubator for 22 - 24 hours. **NOTE:** Ensure a high-humidity (≥ 85%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

8.) For greater convenience on Day 2, retrieve Detection Substrate 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.) 30 minutes before intending to quantify PXR activity, remove Detection Substrate from the refrigerator and place them in a low-light area so that it may equilibrate to room temperature. Gently invert the tube several times to ensure an homogenous solution. **NOTE:** Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.

10.) Set the plate-reader to "luminescence" mode. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, or less.

11.) Following 22 - 24 hours of incubation add 15 µl of Detection Substrate to each well of the assay plate. **NOTE:** Perform manual reagent transfers 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 manual 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.

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.
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