Human Constitutive Androstane Receptor (isoform 1) (NR1I3, CAR1)
Reporter Assay System
3x 32 Assays in 96-well Format
Product # IB00911-32

Technical Manual (version 7.1)

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Human CAR1 Reporter Assay System
3x 32 Assays in 96-well Format

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I. Description

• Constitutive Androstane Receptors •

The family of human Constitutive Androstane Receptors (CAR, NR1I3) regulate the expression of genes involved in xenobiotic metabolism and transport in the liver, including CYP2B and 3A4, UGT1 and MDR. Studies from mouse models show that CAR is also involved in bile acid, thyroid hormone and HDL homeostasis. The human CAR gene is subject to numerous alternative splicing events during pre-mRNA processing. The 348 amino acid isoform 1 of human CAR (CAR1) is encoded by 9 exons comprising the DNA binding domain (DBD), hinge region, and a ligand binding domain (LBD). The primary sequence of CAR2 differs from CAR1 in that it contains a four amino acid (VSPT) insert, whereas CAR3, which is the predominant isoform expressed in the liver, contains a distinct five amino acid (APYLT) insert.

These small sequence variations confer great functional complexity to the human CAR1, 2, and 3 isoforms, including distinct ligand utilization and activation profiles. True to its name, CAR1 is constitutively active, but can be further regulated through ligand interactions, mainly via inverse-agonism. PK11195, clotrimazole, androstane, and 2-ethylhexyl diphenyl phosphate (EDP) exhibit moderate inverse-agonism of CAR1, but show no (or very low) activity against the other CAR isoforms. Unlike CAR1, CAR2 and CAR3 are not constitutively active, showing ligand-dependent activation of reporter genes linked to genetic response elements derived from CYP2B6 or CYP3A4 promoters.

Diethylhexyl phthalate (DEHP) is a strong agonist of CAR2, but has no activity towards CAR1 or CAR3. Conversely, 6-(4-chlorophenyl)imi-dazo[2,1-b]thiazole-5-carbaldehyde O-3,4-dichloroben-zyl)oxime (CITCO) is an exceptionally potent agonist of CAR3, but exhibits no activity against CAR1 or CAR2.

Interestingly, distinct activation profiles and ligand preferences are also a feature of mouse CAR (inducible activation) and rat CAR (constitutive activity). For example, 1,4-bis[3,5-dichloropyridyloxy] benzene (TCPOBOP) and meclizine are potent agonists of mouse CAR, but exhibit no activity to rat CAR or the human CAR isoforms.

It is noteworthy, and a source of experimental confusion, that a number of xenobiotics characterized as activators of human CAR (including phenobarbital) actually modulate the receptor's activity via indirect mechanisms. In other words, such chemicals do not directly bind to CAR, rather, they impact the activity of upstream regulatory mechanisms that impinge on CAR activity. Hybrid nuclear receptors in which the native N-terminal DNA binding domain (DBD) has been substituted with the GAL4 DBD, such as is used in this reporter assay kit, likely will not be responsive to chemical modulators that act through indirect mechanisms.

The expression of human CAR1, 2 and 3 isoforms with their unique activation profiles, disparate responses to xenobiotics, and cross-species differences, can challenge the interpretation of bioactivity profiling data. However, given the importance of CAR activity in predicting drug-drug and drug-nutrient interactions, it is an endeavor worth undertaking.

**The Human CAR1 Assay System**

This assay utilizes proprietary non-human mammalian cells that express isoform 1 of Human Constitutive Androstane Receptor (NR1I3), a ligand-dependent transcription factor referred to as CAR1. These reporter cells utilize a modified version of human CAR1 in which the N-terminal DNA binding domain (DBD) has been replaced with the GAL4-DBD. The human CAR1 ligand binding domain (LBD) is unaltered and fully functional. The reporter cells also incorporate a luciferase cDNA functionally linked to the GAL4-upstream activation sequence (UAS). Thus, quantifying expressed luciferase activity provides a sensitive surrogate measure of changes in CAR1 activity resulting from direct interaction between a treatment compound and the nuclear receptor.

Because this assay system expresses a GAL4-DBD + hCAR1 LBD hybrid receptor, the bioactivity of activators that act through indirect mechanisms (such as those that alter the phosphorylation status of the native N-terminal amino acid sequence of CAR1) may be dampened or go undetected.

Human CAR1 exhibits constitutive activity, but its activity can be further regulated through direct ligand interactions. The primary application of this reporter assay system is in the screening of test samples to quantify any functional inverse-agonist activity that they may exert on human CAR1.

Reporter Cells are prepared using INDIGO’s proprietary CryoMite™ process. This cryopreservation method yields high cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for intermediate spin-and-wash steps, viability determinations, or cell titer adjustments.

INDIGO Bioscience’s Nuclear Receptor Reporter Assays are all-inclusive cell-based assay systems. In addition to CAR1 Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, a positive-control inverse-agonist, Luciferase Detection Reagent, and a cell culture-ready assay plate.

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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 Mg²⁺-dependent reaction that consumes O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PPi, CO₂, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer, and is reported in terms of Relative Light Units (RLU’s).

INDIGO’s Nuclear Receptor Reporter 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. However, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup, and are considered to be 'single-use' reagents.

Assay Scheme

**Figure 1.** Assay workflow. *In brief,* 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 CAR-1 (NR1I3): Inverse-agonist assays**

![Graph showing dose-response analysis](image)

**Figure 2. Dose-response analyses of the Human CAR1 Assay.**

Dose-response analyses of CAR1 Reporter Cells were performed using reference inverse-agonists PK11195 (provided), clotrimazole (Tocris), and EDP (2-ethylhexyl diphenyl phosphate; Sigma) according to the protocol provided in this Technical Manual. Reporter Cells were treated with 8 assay concentrations (n ≥ 6) of respective compounds, each generated in 4-fold decrements as described in Appendix 1. Treatment media were removed after 24 hr and LDR was applied to the cells. Luminescence was quantified using a GloMax-Multi+ luminometer. Average relative light units (RLU) +/- standard deviation (SD) and Fold-Reduction values were determined for each treatment concentration. Z' values were calculated as described by Zhang, et al. (1999). Non-linear regression analyses and IC50 determination were performed using GraphPad Prism software. The same data are plotted in terms of (a) RLU vs. [Cmpd], and (b) Fold-Reduction vs. [Cmpd]. The high Z' values and fold-reductions in CAR1 activities confirm the robust performance of this CAR1 Reporter Assay System, and demonstrate its suitability for use in HTS applications.

Z' = 1-[3*(SDReference max. + SDVehicle) / (RLUVehicle - RLUReference max)]

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II. Product Components & Storage Conditions

This Human CAR1 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 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>CAR1 Reporter Cells</td>
<td>3 x 0.60 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>PK11195, 15 mM (in DMSO)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>(reference inverse-agonist for CAR1)</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>
<td>1</td>
<td>ambient</td>
</tr>
<tr>
<td>Snap-in, 8-well strips (white, sterile, cell-culture ready)</td>
<td>12</td>
<td>ambient</td>
</tr>
</tbody>
</table>

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).

**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.

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

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

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

Total DMSO carried over into assay reactions should never exceed 0.4%.

Note that, in **Step 6**, 100 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 CAR1 Assay System includes a 15 mM stock solution of **PK11195**, an *inverse-agonist* of CAR1 that may be used as a reference. The following 7-point concentration range, presented in 4-fold decrements, provides a complete inverse-agonist dose-response without cytotoxic effects: 30000, 7500, 1875, 469, 117, 29.3, and 7.32 nM, 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.** Sanitize the outside surface of the tube with a 70% alcohol swab.
5.) 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.

**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 (100 µl/well) into a clear 96-well cell culture treated assay plate, followed by 100 µl/well of CSM (as in Step 6). 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% 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 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 CAR1 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.) 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 is NOT advised. Do not touch the well bottom, or run the tip of the aspiration device around the bottom circumference of the assay well. Such practices will result in destruction of the reporter cells and greatly increased well-to-well variability. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using an 8-pin manifold (e.g., Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus.

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

14.) Quantify luminescence.
V. Related Products

**Human CAR1 Assay Products**

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00911-32</td>
<td>Human CAR1 Reporter Assay System 3x 32 assays in 96-well format</td>
</tr>
<tr>
<td>IB00911</td>
<td>Human CAR1 Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB00912</td>
<td>Human CAR1 Reporter Assay System 1x 384-well format assays</td>
</tr>
</tbody>
</table>

**Human CAR3 Assay Products**

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00901-32</td>
<td>Human CAR3 Reporter Assay System 3x 32 assays in 96-well format</td>
</tr>
<tr>
<td>IB00901</td>
<td>Human CAR3 Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB00902</td>
<td>Human CAR3 Reporter Assay System 1x 384-well format assays</td>
</tr>
</tbody>
</table>

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

**LIVE Cell Multiplex (LCM) Assay**

<table>
<thead>
<tr>
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<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCM-01</td>
<td>Reagent volumes sufficient to perform <strong>96</strong> Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well NR assay plates</td>
</tr>
<tr>
<td>LCM-05</td>
<td>Reagents in 5x-bulk volume to perform <strong>480</strong> Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well NR assay plates</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagents in 10x-bulk volume to perform <strong>960</strong> Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well NR assay plates</td>
</tr>
</tbody>
</table>

Please refer to INDIGO Biosciences website for updated product offerings.  
www.indigobiosciences.com
**VI. Limited Use Disclosures**

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic 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 PK11195 reference inverse-agonist, and the setup of a CAR1 dose-response assay.

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1 For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.
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**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</td>
<td>100 μl / well 9.6 ml / plate</td>
<td>~ 2.4 ml</td>
</tr>
<tr>
<td>(prepared from kit components)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDR 12 ml (prepared from kit components)</td>
<td>100 μl / well 9.6 ml / plate</td>
<td>~ 2.4 ml</td>
</tr>
</tbody>
</table>

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**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 CAR-1 (NR1I3): Inverse-agonist assays

Figure 2. Dose-response analyses of the Human CAR1 Assay.

Dose-response analyses of CAR1 Reporter Cells were performed using reference inverse-agonists PK11195 (provided), clotrimazole (Tocris), and EDP (2-ethylhexyl diphenyl phosphate; Sigma) according to the protocol provided in this Technical Manual. Reporter Cells were treated with 8 assay concentrations (n ≥ 6) of respective compounds, each generated in 4-fold decrements as described in Appendix 1. Treatment media were removed after 24 hr and LDR was applied to the cells. Luminescence was quantified using a GloMax-Multi+ luminometer. Average relative light units (RLU) +/- standard deviation (SD) and Fold-Reduction values were determined for each treatment concentration. Z’ values were calculated as described by Zhang, et al. (1999). Non-linear regression analyses and IC50 determination were performed using GraphPad Prism software. The same data are plotted in terms of (a) RLU vs. [Cmpd], and (b) Fold-Reduction vs. [Cmpd]. The high Z’ values and fold-reductions in CAR1 activities confirm the robust performance of this CAR1 Reporter Assay System, and demonstrate its suitability for use in HTS applications.5


\[
Z' = 1 - \frac{3 \times (SD^{Reference \ max.} + SD^{Vehicle})}{(RLU^{Vehicle} - RLU^{Reference \ max.})}
\]
II. Product Components & Storage Conditions

This Human CAR1 Reporter Assay System contains materials to perform assays in a single 96-well assay plate.

The aliquot of 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>• CAR1 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>• PK11195, 15 mM (in DMSO) (reference agonist for CAR1)</td>
<td>1 x 30 µL</td>
<td>-20°C</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 assay plate</td>
<td>1</td>
<td>ambient</td>
</tr>
</tbody>
</table>

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).

**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.

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

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

2.) **Prepare dilutions of treatment compounds:** 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 CAR1 Reporter Assay System includes a 15 mM stock solution of **PK11195**, an **inverse-agonist** of CAR1 that may be used as a reference. The following 7-point concentration range, presented in 4-fold decrements, provides a complete inverse-agonist dose-response without cytotoxic effects: 30000, 7500, 1875, 469, 117, 29.3, and 7.32 nM, 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.) 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 96-well 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 (100 µl/well) into a clear 96-well cell culture treated assay plate, followed by 100 µl/well of CSM (as in **Step 6**). 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% 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 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 FXR 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, 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.) 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 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

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human CAR1 Assay Products</strong></td>
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</tr>
<tr>
<td>IB00911-32</td>
<td>Human CAR1 Reporter Assay System 3x 32 assays in 96-well format</td>
</tr>
<tr>
<td>IB00911</td>
<td>Human CAR1 Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB00912</td>
<td>Human CAR1 Reporter Assay System 1x 384-well format assays</td>
</tr>
<tr>
<td><strong>Human CAR2 Assay Products</strong></td>
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<tr>
<td>IB00921-32</td>
<td>Human CAR2 Reporter Assay System 3x 32 assays in 96-well format</td>
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<td>Human CAR2 Reporter Assay System 1x 96-well format assay</td>
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<tr>
<td>IB00922</td>
<td>Human CAR2 Reporter Assay System 1x 384-well format assays</td>
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<tr>
<td><strong>Human CAR3 Assay Products</strong></td>
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<td>R00911</td>
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<tr>
<td><strong>LIVE Cell Multiplex (LCM) Assay</strong></td>
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<tr>
<td>LCM-01</td>
<td>Reagent volumes sufficient to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well 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 plate formats</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagents in 10x-bulk volume to perform 960 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well plate formats</td>
</tr>
</tbody>
</table>

Please refer to INDIGO Biosciences website for updated product offerings.

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**APPENDIX 1**

Example scheme for the serial dilution of PK11195 reference inverse-agonist, and the setup of a CAR1 dose-response assay.