Human Constitutive Androstane Receptor (isoform 1)

(NR1I3, CAR1)

Reporter Assay System

96-well Format Assays
Product # IB00911-32

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Technical Manual
(version 7.2)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

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

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[1]. The human CAR gene is subject to numerous alternative splicing events during pre-mRNA processing[2]. 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[1].

These small sequence variations confer great functional complexity to the human CAR1, 2, and 3 isoforms, including distinct ligand utilization and activation profiles[4]. 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[1]. Di-ethylhexyl phthalate (DEHP) is a strong agonist of CAR2[3], 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[2-(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.

1. Auerbach, S. S., Stoner, M. A., Su, S. & Omiecinski, C. J. Retinoid X receptor-alpha-dependent transactivation by a naturally occurring structural variant of human constitutive androstane receptor (NR1I3). Mol Pharmacol 68, 1239-1253 (2005).

2. Auerbach, S. S., Ramsden, R., Stoner, M. A., Verlinde, C., et al. Alternatively spliced isoforms of the human constitutive androstane receptor. Nucleic Acids Res 31, 3194-3207 (2003).

3. DeKeyser, J. G., Stagliano, M. C., Auerbach, S. S., Prabhu, K. S., et al. Di(2-ethylhexyl) phthalate is a highly potent agonist for the human constitutive androstane receptor splice variant CAR2. Mol Pharmacol 75, 1005-1013 (2009).

4. Auerbach, S. S., Dekeyser, J. G., Stoner, M. A. & Omiecinski, C. J. CAR2 displays unique ligand binding and RXRalpha heterodimerization characteristics. Drug Metab Dispos 35, 428-439 (2007).

▪ 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 bio-activity 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 CAR 1 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 cryo-preservation method yields high cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for intermediate spin-and-wash steps, viability determinations, or cell titer adjustments.

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.

▪ 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 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.

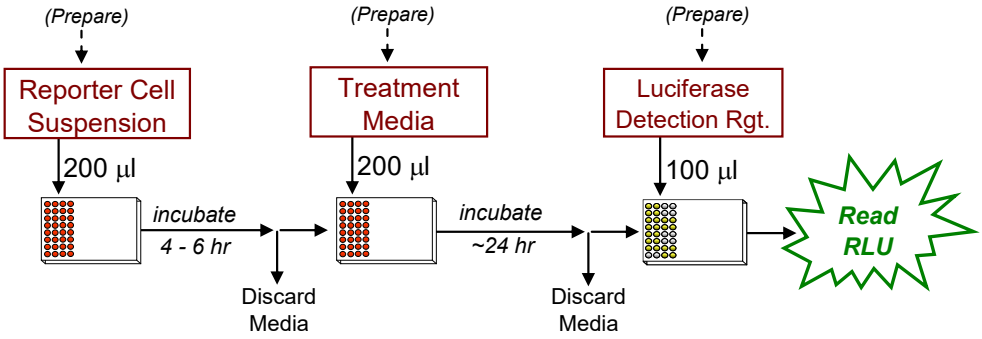
▪ Preparation of Test Compounds ▪

Test compounds are typically solvated at high-concentration in DMSO and stored frozen as master stocks. Immediately prior to setting up an assay, the master stocks are serially diluted using **Compound Screening Medium (CSM**; as described in *Step 7*) to achieve the desired assay concentrations. Do not use DMSO to further dilute test compound solutions. This method of dilution avoids the significant 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%.

▪ 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, 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.



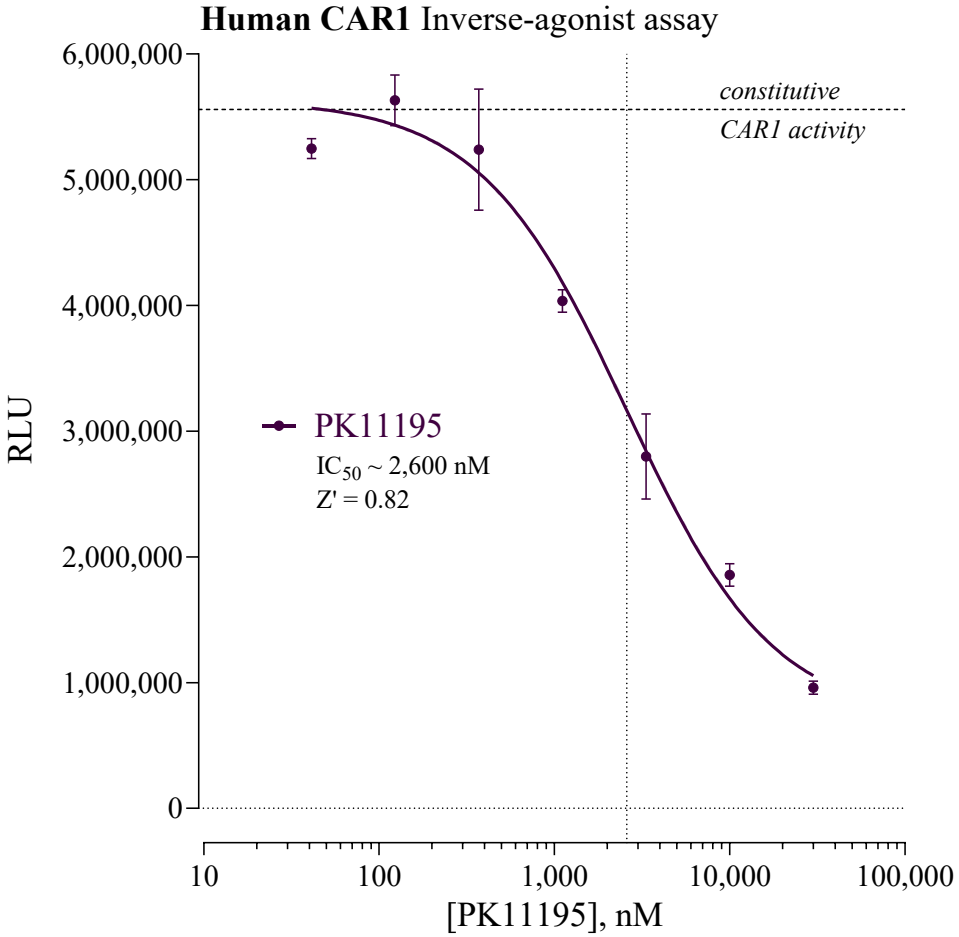


Figure 2. Dose-response analyses of the Human CAR1 Assay.
Dose-response analyses of CAR1 Reporter Cells were performed using reference inverse-agonist PK11195 (provided) according to the protocol provided in this Technical Manual. Reporter Cells were treated with seven concentrations ($n \geq 3$). Average relative light units (RLU) +/- standard deviation (SD) were determined for each treatment concentration. Z' values were calculated as described by Zhang, *et al*. (1999)[5]. Non-linear regression analyses and IC_{50} determination were performed using GraphPad Prism software. 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]

[5] Zhang JH, Chung TD, Oldenburg KR. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen.:**4** (2), 67-73.
$Z' = 1-[3*(SD^{Reference\ max.} + SD^{Vehicle}) / (RLU^{Vehicle} - RLU^{Reference\ max.})]$

II. Product Components & Storage Conditions

This Human CAR1 assay kit 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 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.

Kit Components	***Amount***	***Storage Temp.***
▪ CAR1 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
▪ PK11195, 15 mM (in DMSO) (reference inverse-agonist for CAR1)	1 x 30 µL	-20°C
▪ Detection Substrate	3 x 2.0 mL	**-80°C**
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate frame	1	ambient
▪ Snap-in, 8-well strips (white, sterile, collagen-coated wells)	12	**-80°C**

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

▪ cell culture-rated laminar flow hood.
▪ 37°C, humidified 5% CO_2 incubator for mammalian cell culture.
▪ 37°C water bath.
▪ 70% alcohol wipes
▪ 8- *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).
▪ *Optional:* clear 96-well assay plate, collagen-coated, for viewing cells on Day 2.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-11* are performed on **Day 1**, requiring less than 2 hours of bench work and a 4 hr incubation step to complete. *Steps 12-17* 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 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: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. W*ithout 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.

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 disperse cell aggregates and gain a homogenous cell suspension. Dispense **200 µl / well** of cell suspension into the assay plate.

NOTE 4.1: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling during the dispensing period. Likewise, take care to dispense uniform volumes across the assay plate.

NOTE 4.2: Users sometimes wish to examine the cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear 96-well cell culture treated assay plate. Continue to process the assay plate in identical manner to the white assay plate.

5.) Pre-incubate reporter cells: Place the assay plate into a 37°C, ≥ 85% humidity, 5% CO_2 incubator for 4 - 6 hours.

Near the end of the 4-6 hour pre-incubation period:

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

7.) ***a.*** **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 carried over into assay reactions should never exceed 0.4%.

b. **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 3-fold decrements, provides a complete inverse-agonist dose-response without cytotoxic effects: 30000, 1000, 3333, 1111, 370, 123, and 41.2 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

8.) ***At the end of the cell pre-incubation period:*** **Discard the culture media**.
Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media is NOT advised. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using an 8-pin manifold (*e.g.,* Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus. Do *not* touch the well bottom or run the tip of the aspiration device around the bottom circumference of the assay well. Such practices will result in destruction of the cells and greatly increased well-to-well variability.

9.) Dispense **200 µl** of each treatment media into appropriate wells of the assay plate.

10.) 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.

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. 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.

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*
Human CAR1 Assay Products	
IB00911-32	Human CAR1 Reporter Assay System 3x 32 assays in 96-well format
IB00911	Human CAR1 Reporter Assay System 1x 96-well format assay
IB00912	Human CAR1 Reporter Assay System 1x 384-well format assays
Human CAR2 Assay Products	
IB00921-32	Human CAR2 Reporter Assay System 3x 32 assays in 96-well format
IB00921	Human CAR2 Reporter Assay System 1x 96-well format assay
IB00922	Human CAR2 Reporter Assay System 1x 384-well format assays
Human CAR3 Assay Products	
IB00901-32	Human CAR3 Reporter Assay System 3x 32 assays in 96-well format
IB00901	Human CAR3 Reporter Assay System 1x 96-well format assay
IB00902	Human CAR3 Reporter Assay System 1x 384-well format assays
Mouse CAR Assay Products	
M00901-32	Mouse CAR Reporter Assay System 3x 32 assays in 96-well format
M00901	Mouse CAR Reporter Assay System 1x 96-well format assay

LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in **5x bulk volume** to perform **480** Live Cell Assays performed in 5 x 96-well assay plates
LCM-10	Reagent in **10x bulk volume** to perform **960** Live Cell Assays performed in 10 x 96-well assay plates
INDIGlo Luciferase Detection Reagent	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents in 10 mL, 25 mL, 50 mL, and 500 mL volumes

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

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VI. Limited Use Disclosures

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

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

APPENDIX 1

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