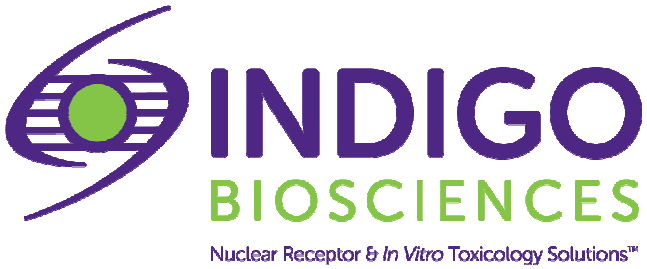

Human Constitutive Androstane Receptor (isoform 3)

(NR1I3, CAR, CAR3)

Reporter Assay System

96-well Format Assays
Product # IB00901

▪

Technical Manual
(version 7.1b)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human CAR3 Reporter Assay System
96-well Format Assays

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 CAR3 Assay System ▪

This nuclear receptor assay system utilizes proprietary non-human mammalian cells engineered to provide constitutive, high-level expression of **Human Constitutive Androstane Receptor**, **isoform 3** (NR1I3), a ligand-dependent transcription factor commonly referred to as **CAR3**. These reporter cells utilize a modified version of human CAR3 in which the native N-terminal DNA binding domain (DBD) has been replaced with that of the GAL4-DBD. The human CAR3 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 CAR3 activity resulting from direct interaction between a treatment compound and the nuclear receptor.

Because this assay system expresses the [GAL4-DBD + hCAR3 LBD] hybrid receptor, the activity of modulators that act through indirect mechanisms (such as those that alter the phosphorylation status of the native N-terminal amino acid sequence of the CARs) may be dampened or go undetected.

Contrary to its name, human CAR3 is *not* constitutively active. Rather, isoform 3 of CAR exhibits ligand-dependent activation. Hence, the primary application of this reporter assay system is in the screening of test compounds to quantify any functional activity, either *agonist o*r *antagonist*, that they may exert on human CAR3.

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 CAR3 Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, a positive-control 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 ▪

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.

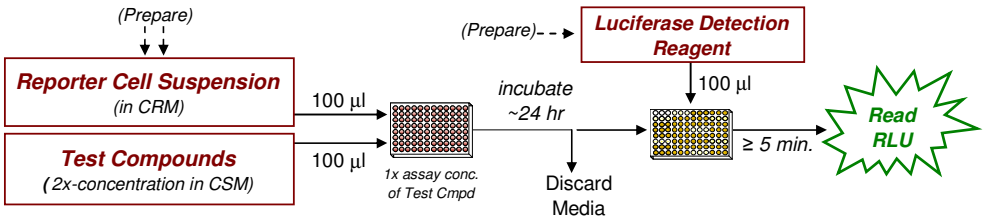
▪ Considerations for Automated Dispensing ▪

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.

Stock Reagent & Volume provided	**Volume to be Dispensed** *(96-well plate)*	**Excess rgt. volume available for instrument dead volume**
Reporter Cell Suspension 12 ml *(prepared from kit components)*	100 µl / well 9.6 ml / plate	~ 2.4 ml
LDR 12 ml *(prepared from kit components)*	100 µl / well 9.6 ml / plate	~ 2.4 ml

▪ 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.



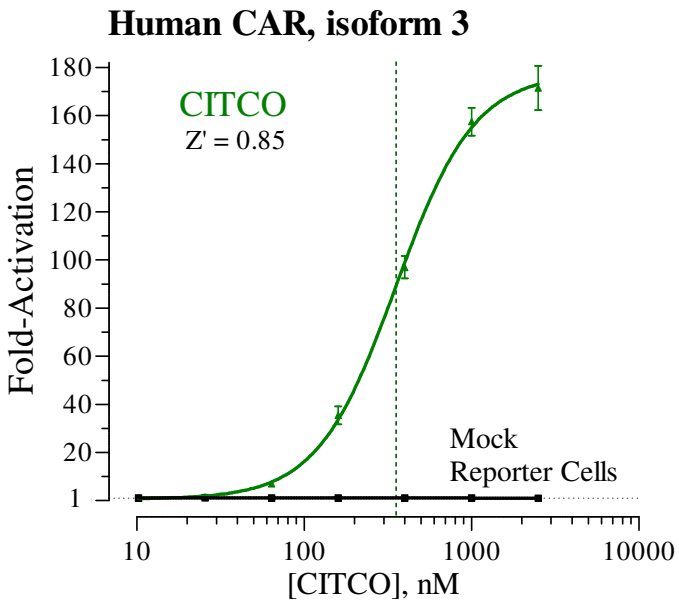


Figure 2a. CITCO agonist dose-response of the CAR3 Assay.

Dose-response analyses of CAR3 Reporter Cells were performed according to the protocol provided in this Technical Manual. In addition, to assess the level of background signal contributed by non-specific factors that may cause activation of the luciferase reporter gene, "mock" reporter cells were prepared to contain only the luciferase vector (mock reporter cells are not provided with assay kits). CAR3 Reporter Cells and mock reporter cells were identically treated with CITCO using an assay concentration range generated in 2.5-fold increments (as described in Appendix 1): 2500, 1000, 400, 160, 64.0, 25.6, 10.2 and 0 nM. Luminescence was quantified using a GloMax-Multi+ luminometer. Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration ($n \geq 6$). Signal-to-background (S/B) and Z' values were calculated as described by Zhang, *et al*. (1999)[1]. Non-linear regression analyses and EC_{50} determination were performed using GraphPad Prism software.

RESULTS: CAR3 reporter cells treated with 2500 nM CITCO yielded a S/B of ~ 170 and a corresponding Z'= 0.85. Similarly treated mock reporter cells demonstrate no significant background luminescence (≤ 0.05% that of the reporter cells at EC_{Max}). Thus, luminescence results strictly through ligand-activation of the human CAR3 expressed in these reporter cells. These data confirm the robust performance of this CAR3 Reporter Assay System, and demonstrate its suitability for use in HTS applications.[1]

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

[1] Zhang JH, Chung TD, Oldenburg KR. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. J Biomol Screen.:**4** (2), 67-73.

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

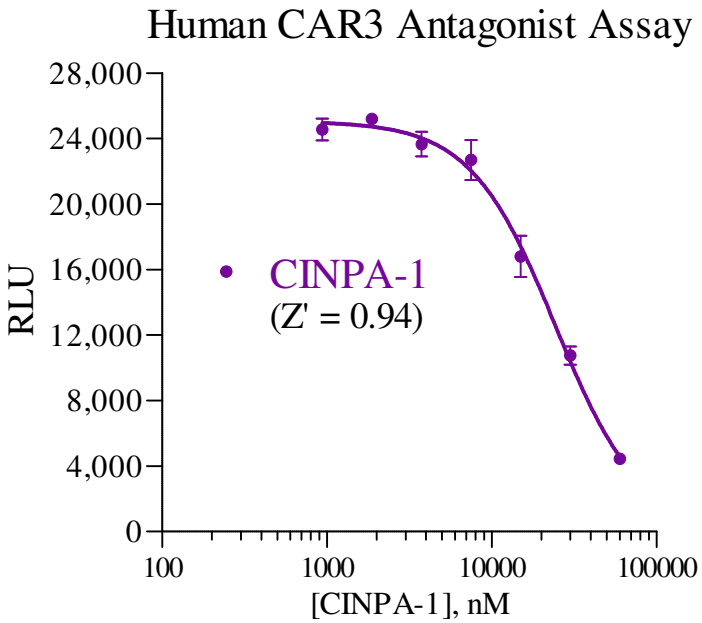


Figure 2b. CINPA-1 dose-dependent inhibition of CAR3

Human CAR3 Reporter Cells were treated with ~EC_{80} of CITCO and then challenged with 2-fold incremental concentrations of CINPA-1 (Tocris). The following concentrations were tested: 60, 30, 15, 7.5, 3.75, 1.88, 0.938 and 0 µM CINPA-1.

II. Product Components & Storage Conditions

This Human CAR3 Reporter Assay System contains materials to perform assays in a single 96-well assay plate.

The aliquot of CAR3 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.

Kit Components	***Amount***	***Storage Temp.***
▪ CAR3 Reporter Cells	1 x 2.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ CITCO*, 4.0 mM (in DMSO) (reference agonist for CAR3)	1 x 30 µL	-20°C
▪ Detection Substrate	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well assay plate (white, sterile, cell-culture ready)	1	ambient

* 6-(4-chlorophenyl)imi-dazo[2,1-b] thiazole-5-carbaldehyde O-3,4-dichloroben-zyl) oxime (CITCO)

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).

▪ 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 CAR3 Reporter Assay System kit includes a 4.0 mM stock solution of **CITCO**, a potent agonist of CAR3 that may be used to setup antagonist-mode assays. 400 nM CITCO typically approximates EC_{55} 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 CAR3 Reporter Assay System kit includes a 4.0 mM stock solution of **CITCO**, a potent agonist of CAR3 that may be used as a reference compound. The following 7-point treatment series, with concentrations presented in 2.5-fold decrements, provides a suitable dose-response: 2500, 1000, 400, 160, 64, 25.6, and 10.2 nM (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. 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 96-well 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. 8). Dispense 100 µl of cell suspension into each well of the 96-well 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 the addition of 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% 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.

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 CAR3 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

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
Rat CAR Assay Products	
R00911-32	Rat CAR Reporter Assay System 3x 32 assays in 96-well format
R00911	Rat CAR Reporter Assay System 1x 96-well format assay
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 plate formats
LCM-05	Reagents in 5x-bulk volume to perform **480** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well plate formats
LCM-10	Reagents in 10x-bulk volume to perform **960** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well plate formats

Please refer to INDIGO Biosciences website for updated product offerings.

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VI. Limited Use Disclosures

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

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

APPENDIX 1

Example scheme for the serial dilution of CITCO reference agonist, and the setup of a CAR3 dose-response assay.