

**Human Constitutive
Androstane Receptor, isoform 2 (CAR 2)
(NR1I3 isoform 2)
Reporter Assay System**

3x 32 Assays in 96-well Format
Product # IB00921-32

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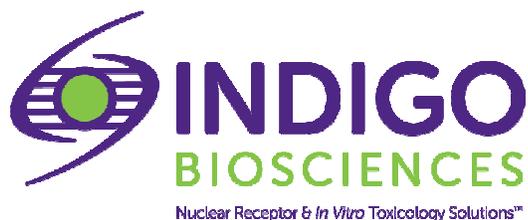
Technical Manual
(*version 7.2b*)

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Human CAR2 Reporter Assay System 96-well Format Assays

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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 homeostasis¹. The human CAR gene is subject to numerous alternative splicing events during pre-mRNA processing². 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) insert¹.

These small sequence variations confer great functional complexity to the human CAR1, 2, and 3 isoforms, including distinct ligand utilization and activation profiles⁴. 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, CAR isoforms 2 and 3 are *not* constitutively active, showing ligand-dependent activation of reporter genes linked to genetic response elements derived from CYP2B6 or CYP3A4 promoters¹. Di-ethylhexyl phthalate (DEHP) is a strong agonist of CAR2³, but has no activity towards CAR1 or CAR3. Conversely, 6-(4-chlorophenyl)imi-dazo[2,1-b] thiazole-5-carbaldehyde O-3,4-dichloroben-zyloxi-mine (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, 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 RXR alpha heterodimerization characteristics. *Drug Metab Dispos* 35, 428-439 (2007).

▪ The Human CAR2 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 2** (NR1I3), a ligand-dependent transcription factor commonly referred to as **CAR2**. These reporter cells utilize a modified version of human CAR2 in which the native N-terminal DNA binding domain (DBD) has been replaced with that of the yeast GAL4-DBD. The CAR2 ligand binding domain (LBD) is unaltered and fully functional. The reporter cells also incorporate a luciferase cDNA functionally linked to the yeast GAL4-upstream activation sequence (UAS). Thus, quantifying expressed luciferase activity provides a sensitive surrogate measure of changes in CAR2 activity resulting from direct interaction between a treatment compound and the nuclear receptor.

Because this assay system expresses the [GAL4-DBD + hCAR2 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) are unlikely to be detected.

Contrary to its name, human CAR2 is *not* constitutively active. Rather, isoform 2 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 or antagonist*, that they may exert on human CAR2.

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 viability determinations or cell titer adjustments.

INDIGO Bioscience's Nuclear Receptor Reporter Assays are all-inclusive cell-based assay systems. In addition to CAR2 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 Assay kits feature a luciferase detection reagent specially formulated to provide stable light emission between 5 and 90+ minutes after initiating the luciferase reaction. Incorporating a 5 minute reaction-rest period ensures that light emission profiles attain maximal stability, thereby allowing assay plates to be processed in batch. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.

▪ Preparation of Test Compounds ▪

NOTE: This Human CAR2 assay protocol recommends a dilution method that differs significantly from INDGIO's other CAR assay protocols that some users may be accustomed to. Using CSM exclusively to generate serial dilutions of the hydrophobic reference agonist DEHP will yield inaccurate dose-response metrics.

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 one of two alternative strategies:

The following is the preferred method of test compound dilutions for the CAR2 assay. This method is presented in Step 7 of the assay protocol, and depicted in APPENDIX 1 for preparing dilutions of the reference agonist DEHP: DMSO should be used to make initial serial dilutions that generate 1,000x-concentrated stocks for each independent test concentration. CSM is then used as diluent in a 2-step process to generate the final (1x concentration) treatment media by making 1,000-fold dilutions of each DMSO stock.

The final concentration of total DMSO carried over into assay wells should *never* exceed 0.4%. DMSO-induced cytotoxicity can be expected above 0.4% residual solvent in the assay wells.

NOTE: Final treatment media preparations containing high concentrations of extremely hydrophobic test compounds (such as DEHP) will lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that final treatment media are always prepared immediately prior to assay setup and are considered to be 'single-use' reagents.

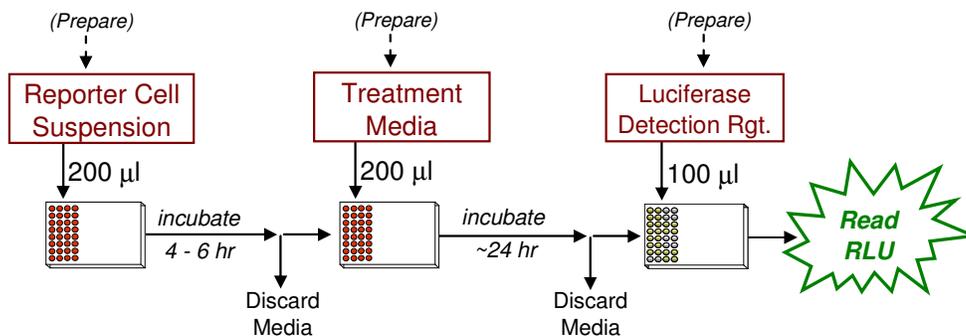
▪ Assay Scheme ▪

Figure 1. Assay workflow.

NOTE: This Human CAR2 assay protocol includes Day 1 steps and dispensed volumes that differ from INDGIO's other CAR assay protocols that some users may be accustomed to; please review the assay workflow, below.

In brief,

- Reporter Cells are dispensed into wells of the assay plate and pre-incubated for 4-6 hours.
- Following the pre-incubation period, culture media are discarded, and 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.



▪ Assay Performance ▪

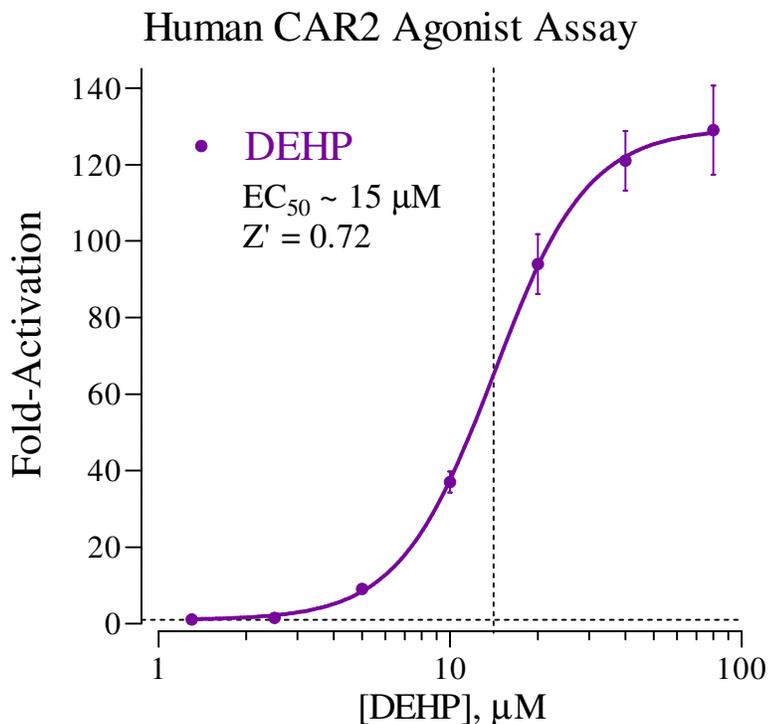


Figure 2. Agonist dose-response analyses of Human CAR2.

Human CAR2 Reporter Cells were treated with the reference agonists DEHP (*bis* 2-ethylhexyl phthalate; provided). Luminescence was quantified and average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration ($n \geq 6$). Fold-activation and Z' values were calculated as described by Zhang, *et al.* (1999)¹. Non-linear regression and EC₅₀ analyses were performed using GraphPad Prism software.

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.

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

II. Product Components & Storage Conditions

This Human CAR2 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ hCAR2 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
▪ DEHP, 120 mM (in DMSO) (reference agonist for hCAR2)	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

III. Materials to be Supplied by the User

The following materials must be provided by the user, and should be made ready prior to initiating the assay procedure:

DAY 1

- container of dry ice (see *Step 2*)
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media basins, sterile.
- sterile 0.2 ml capacity PCR 8 well-strips or individual PCR tubes; multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), *or* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- *Optional*: antagonist reference compound.
- *Optional*: clear 96-well assay plate, cell culture treated, for viewing cells on Day 2.

DAY 2 plate-reading luminometer.

IV. Assay Protocol

Review the entire Assay Protocol before starting. Completing the assay requires an overnight incubation. *Steps 1-11* are performed on **Day 1**, requiring less than 2 hours of 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.

▪ A word about Antagonist-mode assay setup ▪

Receptor inhibition assays expose the Reporter Cells to a constant, sub-maximal concentration (typically between EC₅₀ – EC₈₅) of a reference agonist in combination with varying concentrations of the test compound(s). This CAR2 Assay kit includes a 120 mM stock solution of **DEHP**, an agonist of human CAR2 that may be used to setup antagonist-mode assays. 25 µM DEHP typically approximates EC₈₀ in this cell-based assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

Add the challenge agonist to a bulk volume of **CSM** at an EC₅₀ – EC₈₅ concentration. This medium is then used to prepare serial dilutions of test compounds to achieve the desired respective final assay concentrations. We find that this is an efficient and precise method of setting up CAR2 antagonist assays, and it is the method presented in *Step 7b* of this protocol.

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

1.) Remove the **2 tubes** of **Cell Recovery Medium (CRM)** from freezer storage, thaw and equilibrate to 37°C using a water bath.

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

Second, retrieve **Reporter Cells** from -80°C storage and place them directly into dry ice to transport them to the laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. When ready to begin, transfer the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring **6.4 ml** of pre-warmed CRM into each tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.0 ml per tube.

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 mounted strip-wells.

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% CO₂ incubator for 4 - 6 hours.

6.) Near the end of the 4-6 hour pre-incubation period: Remove **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.

7.) Prepare the Test Compound and Reference Compound treatment media at the desired final assay concentrations. Refer to “*Preparation of Test Compounds*” pg 5 for discussion. APPENDIX 1 provides a depiction of the following dilution strategy:

a.) Use DMSO as the diluent to make intermediate serial dilutions of reference and test compounds. The objective is to use DMSO to prepare 1,000x-concentrated stocks for each independent treatment concentration.

b.) CSM is then used as diluent to generate the final series of (1x concentration) treatment media. Specifically, use CSM to prepare 1,000-fold dilutions of each DMSO concentrated stock. To ensure accurate volume transfers, we recommend using a 2-step dilution method employing serial 1/25-fold and 1/40-fold dilutions (as depicted in APPENDIX 1). NOTE: Some of the 1/25 intermediate stocks may show turbidity. This will not pose a problem. Proceed to the next 1/40 dilution, which will yield non-turbid, DEHP treatment media at their respective final 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%.

a. Agonist-mode assays. This Assay kit includes a 120 mM stock solution of DEHP, a reference agonist of human CAR2. The following 7-point treatment series, prepared in serial 2-fold decrements, provides a complete dose-response: 60, 30, 15, 7.5, 3.75, 1.88, and 0.938 µM. Always include a 'no treatment' control. APPENDIX 1 provides an example for generating such a dilution series.

~ or ~

b. Antagonist-mode assays. When setting up antagonist assays, first supplement a bulk volume of CSM with the challenge agonist DEHP to achieve an EC₅₀ – EC₈₀ concentration (refer to “*A word about antagonist-mode assay setup*”, pg. 8). The agonist-supplemented CSM is then used to generate dilutions of test compound stocks to achieve their final assay concentrations.

8.) (continuing from Step 5) 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 bottoms or run the tip of the aspiration device around the bottom circumference of the assay wells. Such practices will result in destruction of the cells and greatly increased well-to-well variability.

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

10.) 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.

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

<i>Product No.</i>	<i>Product Descriptions</i>
Human CAR2 Assay Kit Products	
IB00921-32	3x 32 Human CAR2 assays; strip-wells in 96-well plate frame
IB00921	1x 96-well format Human CAR2 assays
IB00922	1x 384-well format Human CAR2 assays
Human CAR1 Assay Kit Products	
IB00911-32	3x 32 Human CAR1 assays; strip-wells in 96-well plate frame
IB00911	1x 96-well format Human CAR1 assays
IB00912	1x 384-well format Human CAR1 assays
Human CAR3 Assay Kit Products	
IB00901-32	3x 32 Human CAR3 assays; strip-wells in 96-well plate frame
IB00901	1x 96-well format Human CAR3 assays
IB00902	1x 384-well format Human CAR3 assays
Rat CAR Assay Kit Products	
R00911-32	3x 32 Rat CAR assays; strip-wells in 96-well plate frame
R00911	1x 96-well format Rat CAR assays
Mouse CAR Assay Kit Products	
M00901-32	3x 32 Mouse CAR assays; strip-wells in 96-well plate frame
M00901	1x 96-well format Mouse CAR assays
LIVE Cell Multiplex (LCM) Assay Products	
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 contained in 5 x 96-well assay plates
LCM-10	Reagent in 10x bulk volume to perform 960 Live Cell Assays contained in 10 x 96-well assay plates

Refer to INDIGO Biosciences website for updated product offerings.

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VI. Limited Use Disclosures

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APPENDIX 1

Example scheme for the serial dilution of DEHP reference agonist, and the setup of a Human CAR2 dose-response assay.

