Human Constitutive Androstane Receptor (*isoform 3*)

(NR1I3, CAR, CAR3)

Reporter Assay System

3x32 Assays in 96-well Plate Format
Product # IB00901-32

▪

Technical Manual
(version 7.2i)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human CAR3 Reporter Assay System

3x 32 Assays in 96-well Plate 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[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.

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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 to 10 minutes 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 ▪

Small molecule test compounds are typically solvated in DMSO at high concentrations; ideally 1,000x-concentrated stocks relative to the highest desired treatment concentration in the assay. Using high-concentration stocks minimizes DMSO carry-over into the assay plates. Immediately prior to setting up an assay, the master stocks are serially diluted using one of two alternative strategies:

1.) As described in *Step 7* and depicted in Appendix 1 for the reference agonist CITCO, **Compound Screening Medium (CSM)** is used as the diluent to make serial dilutions of test compounds to achieve the desired assay concentrations.

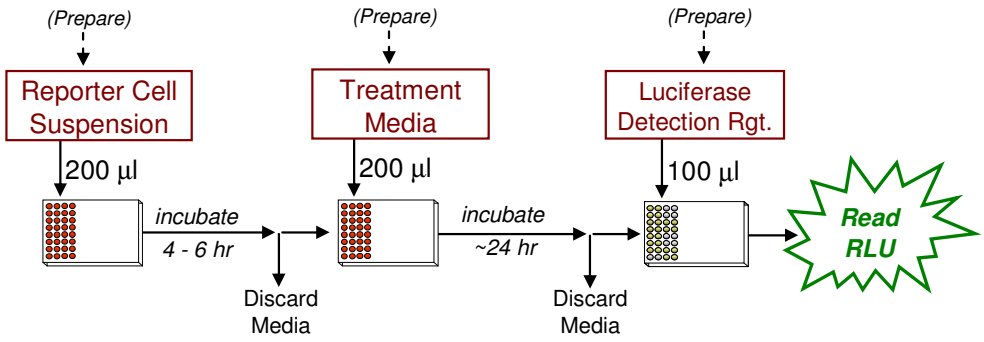
Alternatively, if test compound solubility is expected to be problematic,
2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the concentration of total DMSO carried over into assay wells should not exceed 0.4%. DMSO-induced cytotoxicity can be expected above 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 then considered to be 'single-use' reagents.

▪ 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 treatment media are added. Following 22 - 24 hours incubation, treatment media are discarded, and Luciferase Detection Reagent is added. The intensity of light emission (in terms of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



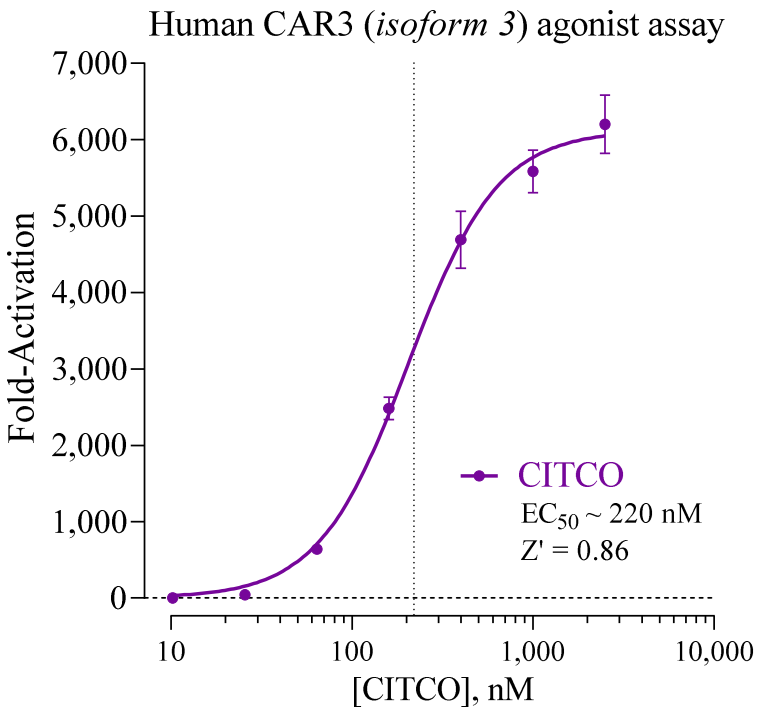


Figure 2. Agonist dose-response of the CAR3 Assay.

Dose-response analysis of CAR3 Reporter Cells was performed using the reference agonist CITCO (provided). CITCO treatment media were prepared using serial 3-fold dilutions, as described in Appendix 1. Luminescence was quantified and average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n = 3). Values of Fold-Activation and Z'[1] were calculated. The plot of 'Fold-Activation *vs.* Log_{10}[CITCO, nM]' and EC50 determination were performed *via* least-squares non-liner regression 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.

$$Z' = 1 - [3*(SD^{CITCO} + SD^{Vehicle}) / (RLU^{CITCO} - RLU^{Vehicle})]$$

II. Product Components & Storage Conditions

This Human CAR-3 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.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in *Step 2* of this protocol.

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first inspect and inventory the individual kit components be sure to first transfer and submerge the tube of reporter cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen, nor can they be maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

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

Kit Components	*Amount*	*Storage Temp.*
▪ CAR-3 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
▪ CITCO, 6.0 mM (in DMSO) (reference agonist for CAR-3)	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	**-20°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 -80°C) 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

▪ dry ice bucket (*Step 2*)
▪ 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-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* sterilized 96 deep-well blocks (*e.g.,* Axygen Scientific, #P-2ML-SQ-C-S), *or* appropriate similar vessel for generating dilution series of reference and test compound(s).
▪ *Optional:* clear 96-well assay plate, sterile, *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.

▪ A word about Antagonist-mode assay setup ▪

When setting up receptor inhibition assays the Reporter Cells are co-treated with a fixed sub-maximal concentration (typically between EC_{50} – EC_{85}) of the reference agonist AND varying concentrations of the test compound(s). This CAR3 Assay kit includes a 6.0 mM stock solution of **CITCO**, a potent agonist of CAR3 that may be used to perform antagonist-mode assays. 500 nM CITCO typically approximates $EC_{70\text{-}80}$ in this cell-based assay and, therefore, is a suitable concentration of agonist to be used when screening test compounds for inhibitory activity.

Add the challenge agonist (CITCO) to a bulk volume of **CSM** at an EC_{50} – EC_{85} concentration. This medium is then used to prepare serial dilutions of test compounds to achieve the desired respective assay concentrations. This is an efficient and precise method of setting up 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 tubes of **Reporter Cells** from -80°C storage and place them directly into dry ice for transport 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.

Third, during the 5 - 10 minutes incubation period, 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.

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

NOTE 4.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'blank' wells (meaning cell-free, but containing 'CSM') must be included in the assay plate to allow quantification of fluorescence background (refer to the LCMA Technical Manual).

NOTE 4.2: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the reservoir during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.

NOTE 4.3: Users sometimes wish to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear, *collagen-coated* 96-well assay plate. Continue to process the clear plate in identical manner to the white assay plate.

5.) Pre-incubate reporter cells. Place the assay plate into a cell culture incubator (37°C, ≥ 70% humidity, 5% CO_2) for 4 - 6 hours.

6.) Near the end of the 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: Use **CSM** to prepare an appropriate dilution series of the reference and test compound stocks. In *Step 9*, **200 µl / well** of the prepared treatment media are dispensed into the strip-wells of the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO carried over into assay reactions should not exceed 0.4%.

***a. Agonist*-mode assays**. This CAR-3 assay kit includes a 6.0 mM stock solution of CITCO, a potent agonist of CAR-3. The following 7-point treatment series, prepared in serial 3-fold decrements, provides a complete dose-response: 6000, 2000, 667, 222, 74.1, 024.7, and 8.23 nM CITCO. 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 CITCO to achieve the desired fixed assay-concentration (refer to "*A word about antagonist-mode assay setup*", pg. 7). This agonist-supplemented CSM is then used to generate dilutions of test compound stocks to achieve the desired assay concentration 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 either a single-tip, or 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, degrade assay performance, 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 (≥ 70%) 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.

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. Program 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.

14.) Immediately before proceeding to *Step 15*, prepare **Luciferase Detection Reagent** (**LDR**): 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 **LDR**. Mix gently to avoid foaming.

15.) Following 22 - 24 hours incubation in treatment media, carefully remove media contents from each well of the assay plate (as before in *Step 8*).

16.) Use an 8-channel pipette to dispense 100 µl of **LDR** to each well of the assay plate. Allow the plate to rest at room temperature for 5 - 10 minutes. Do not shake the plate during this period.

17.) Quantify luminescence.

V. Related Products

Human CAR2 Assay Products	
Product No.	***Product Descriptions***
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
Rat CAR Assay Products	
R00901-32	Rat CAR Reporter Assay System 3x 32 assays in 96-well format
R00901	Rat CAR Reporter Assay System 1x 96-well format assay
Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays in 1x96-well, 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

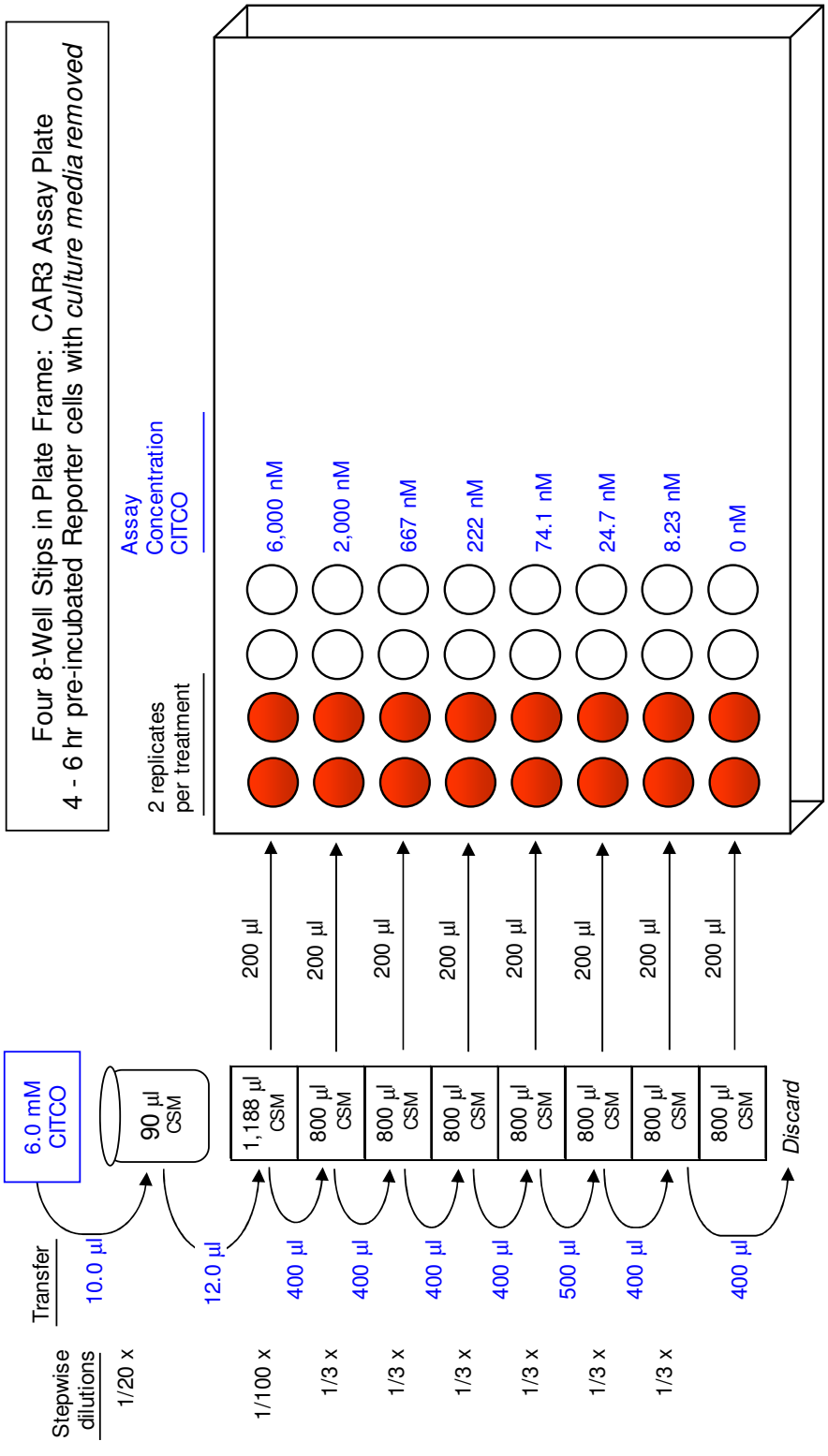
Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic, diagnostic, or contact use in humans or animals.

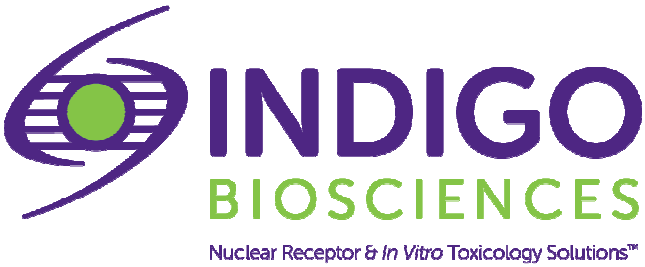
"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA).

Product prices, availability, specifications, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most currently updated version.

APPENDIX 1

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





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2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the concentration of total DMSO carried over into assay wells should not exceed 0.4%. DMSO-induced cytotoxicity can be expected above 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 then considered to be 'single-use' reagents.

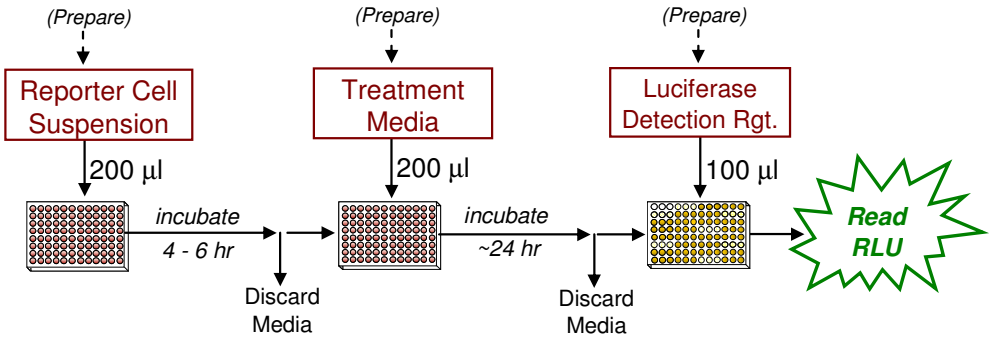
▪ Considerations for Automated Dispensing ▪

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 21 ml *(prepared from kit components)*	200 µl / well 19.2 ml / plate	~ 1.8 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*, 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 treatment media are added. Following 22 - 24 hours 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 ▪

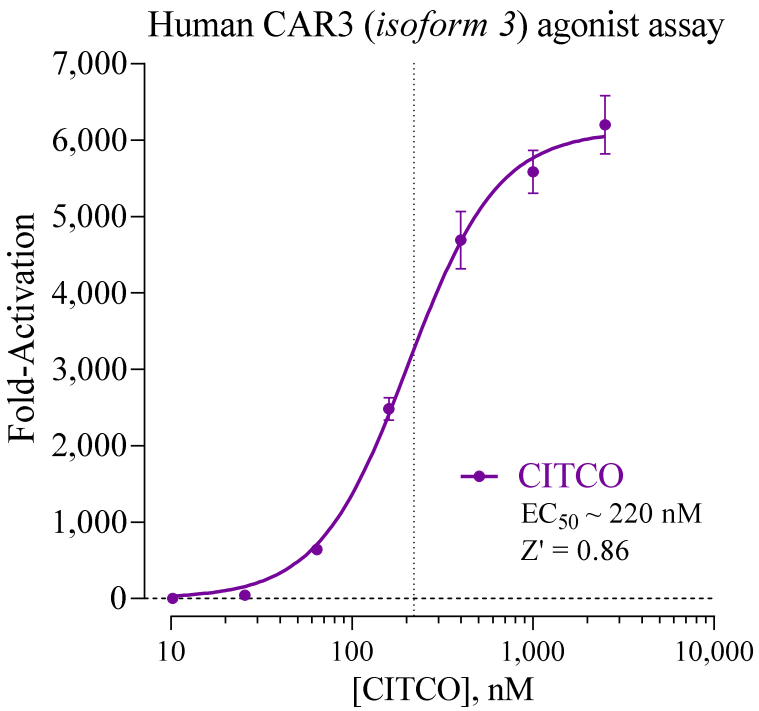


Figure 2. Agonist dose-response of the CAR3 Assay.

Dose-response analysis of CAR3 Reporter Cells was performed using the reference agonist CITCO (provided). CITCO treatment media were prepared using serial 3-fold dilutions, as described in Appendix 1. Luminescence was quantified and average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n = 3). Values of Fold-Activation and Z'[1] were calculated. The plot of 'Fold-Activation *vs.* Log_{10}[CITCO, nM]' and EC50 determination were performed *via* least-squares non-liner regression 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.

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

II. Product Components & Storage Conditions

This Human CAR-3 Assay kit contains materials to perform assays in a single collagen-coated 96-well assay plate.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in *Step 2* of this protocol.

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first inspect and inventory the individual kit components be sure to first transfer and submerge the tube of reporter cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen, nor can they be maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

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

Kit Components	***Amount***	***Storage Temp.***
▪ CAR-3 Reporter Cells	1 x 2.0 mL	-80°C
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ CITCO, 6.0 mM (in DMSO) (reference agonist for CAR-3)	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, *collagen-coated* assay plate (white, sterile, cell-culture ready)	1	**-20°C**

NOTE: This Assay kit contains one 96-well assay plate in which the assay wells have been collagen-coated and dried; the assay plate should be stored frozen (-20°C or colder) until use.

III. Materials to be Supplied by the User

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

DAY 1

▪ dry ice bucket (*Step 2*)
▪ 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-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* sterilized 96 deep-well blocks (*e.g.,* Axygen Scientific, #P-2ML-SQ-C-S), *or* appropriate similar vessel for generating dilution series of reference and test compound(s).
▪ *Optional:* clear 96-well assay plate, sterile, *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.

▪ A word about Antagonist-mode assay setup ▪

When setting up receptor inhibition assays the Reporter Cells are co-treated with a fixed sub-maximal concentration (typically between EC_{50} – EC_{85}) of the reference agonist AND varying concentrations of the test compound(s). This CAR3 Assay kit includes a 6.0 mM stock solution of **CITCO**, a potent agonist of CAR3 that may be used to perform antagonist-mode assays. 500 nM CITCO typically approximates $EC_{70\text{-}80}$ in this cell-based assay and, therefore, is a suitable concentration of agonist to be used when screening test compounds for inhibitory activity.

Add the challenge agonist (CITCO) to a bulk volume of **CSM** at an EC_{50} – EC_{85} concentration. This medium is then used to prepare serial dilutions of test compounds to achieve the desired respective assay concentrations. This is an efficient and precise method of setting up 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 the tube of **Reporter Cells** from -80°C storage, place it directly into a dry ice bucket and transport the cells to the laminar flow hood. When ready, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by transferring 9.5 ml from ***each* of the 2 tubes** of 37°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **21 ml**.

3.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface with a 70% alcohol swab.

4.) Gently invert the tube of Reporter Cells several times to gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an 8-chanel pipette, dispense **200 µl / well** of cell suspension into the assay plate.

NOTE 4.1: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'blank' wells (meaning cell-free, but containing 'CSM') must be included in the assay plate to allow quantification of fluorescence background (refer to the LCMA Technical Manual).

NOTE 4.2: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the reservoir during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.

NOTE 4.3: Users sometimes wish to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear, *collagen-coated* 96-well assay plate. Continue to process the clear plate in identical manner to the white assay plate.

5.) Pre-incubate reporter cells. Place the assay plate into a cell culture incubator (37°C, ≥ 70% humidity, 5% CO_2) for 4 - 6 hours.

6.) Near the end of the 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: Use **CSM** to prepare an appropriate dilution series of the reference and test compound stocks. 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 not exceed 0.4%.

***a. Agonist*-mode assays**. This CAR-3 assay kit includes a 6.0 mM stock solution of CITCO, a potent agonist of CAR-3. The following 7-point treatment series, prepared in serial 3-fold decrements, provides a complete dose-response: 6000, 2000, 667, 222, 74.1, 024.7, and 8.23 nM CITCO. 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 CITCO to achieve the desired fixed assay-concentration (refer to "*A word about antagonist-mode assay setup*", pg. 7). This agonist-supplemented CSM is then used to generate dilutions of test compound stocks to achieve the desired assay concentration series.

8.) At the end of the 4 - 6 hours pre-incubation period, discard the media. The preferred method is to use a 'wrist flick' to eject media 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.

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

NOTE: If well-to-well variation due to 'edge-effects' is a concern this problem *may* be mitigated by dispensing sterile liquid into the *inter-well* spaces of the assay plate. Simply remove 1 tip from the 8-chanel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

10.) Transfer the assay plate into a 37°C, humidified 5% CO_2 incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥70%) 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 **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.

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. Program 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.

14.) Immediately before proceeding to *Step 15*, prepare **Luciferase Detection Reagent** (**LDR**). Combine 'Detection Buffer' and 'Detection Substrate' by pouring-over their entire volumes into a media basin; rock the basin gently to mix the reagent. The resulting volume of LDR is 12 ml.

15.) Following 22 - 24 hours incubation in treatment media, discard the media contents by manually ejecting it into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets.

16.) Add 100 µl of **LDR** to each well of the assay plate. Allow the plate to rest at room temperature for 5 - 10 minutes. Do not shake the assay plate during this period.

17.) Quantify luminescence.

V. Related Products

Human CAR2 Assay Products	
Product No.	***Product Descriptions***
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
Rat CAR Assay Products	
R00901-32	Rat CAR Reporter Assay System 3x 32 assays in 96-well format
R00901	Rat CAR Reporter Assay System 1x 96-well format assay
Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays in 1x96-well, 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.

www.indigobiosciences.com

VI. Limited Use Disclosures

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

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

Product prices, availability, specifications, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most currently updated version.

APPENDIX 1

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