



Mouse Constitutive Androstane Receptor

(nr1i3, mCAR)

Reporter Assay System

96-well Format Assays

Product # M00901

▪

Technical Manual

(version 7.1)

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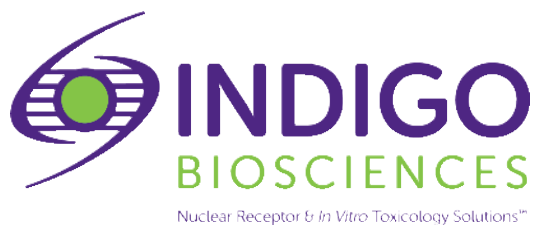
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Mouse CAR Reporter Assay System 96-well Format Assays

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I. Description

▪ The Mouse CAR Assay System ▪

This nuclear receptor assay utilizes proprietary non-human mammalian cells engineered to provide constitutive, high-level expression of **Mouse Constitutive Androstane Receptor**, (nr1i3), a ligand-dependent transcription factor commonly referred to as **mCAR**. These reporter cells utilize a modified version of mCAR in which the native N-terminal DNA binding domain (DBD) has been replaced with that of the GAL4-DBD. The mCAR 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 mCAR activity resulting from direct interaction between a treatment compound and the nuclear receptor.

Because this assay expresses the [GAL4-DBD + mCAR 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) will be dampened or go undetected.

Contrary to its name, mouse CAR is *not* constitutively active, rather, it exhibits ligand-dependent activation. The primary application of this assay is in the screening of test compounds to quantify any functional activity, either *agonist* or *antagonist*, that they may exert on mouse CAR.

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 assay kits are all-inclusive cell-based assay systems. In addition to mCAR 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 assays 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 O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, CO₂, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer, and is reported in terms of Relative Light Units (RLU's).

INDIGO's Nuclear Receptor assays 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.

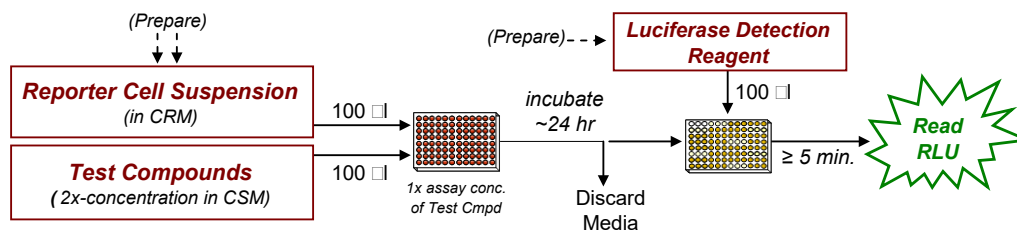
▪ **Considerations for Automated Dispensing** ▪

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 <i>(prepared from kit components)</i>	100 µl / well 9.6 ml / plate	~ 2.4 ml
LDR 12 ml <i>(prepared from kit components)</i>	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.



▪ Assay Performance ▪

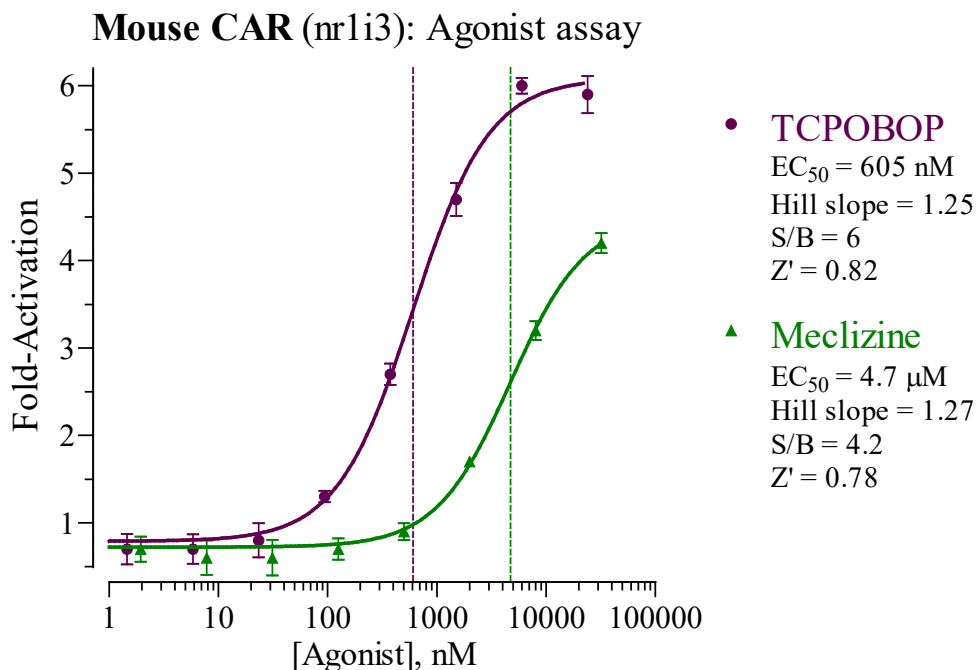


Figure 2. TCPOBOP agonist dose-response of the mCAR Assay.

Dose-response analyses of mCAR Reporter Cells were performed according to the protocol provided in this Technical Manual. mCAR Reporter Cells were treated with the reference agonists TCPOBOP (provided) and Meclizine (Tocris). 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 ($n \geq 6$), and Fold-Activation (*i.e.*, signal-to-background) and Z' values were calculated as described by Zhang, *et al.* (1999)¹. Non-linear regression analyses and EC₅₀ determination were performed using GraphPad Prism software.

RESULTS: mCAR reporter cells treated with 6.0 μM TCPOBOP yielded a S/B of ~ 6 and a corresponding Z'= 0.82. These data confirm the robust performance of this mCAR assay, 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.

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

II. Product Components & Storage Conditions

This Mouse CAR Assay kit contains materials to perform assays in a single 96-well assay plate.

The aliquot of mCAR 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ mCAR 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
▪ TCPOBOP*, 10 mM (in DMSO) (reference agonist for mCAR)	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

* 1,4-Bis(3,5-Dichloro-2-pyridinyloxy)benzene (TCPOBOP; CAS No. 76150-91-9)

III. Materials to be Supplied by the User

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

DAY 1

- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8- or 12-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media basins, sterile.
- sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), or deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- 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 mCAR assay kit includes a 10 mM stock solution of **TCPOBOP**, an agonist of mCAR that may be used to setup antagonist-mode assays. 2.0 μ M TCPOBOP typically approximates EC_{80} in this cell-based 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 μ l of treatment media is combined with 100 μ l 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 aseptic technique.

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

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

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

Note that, in *Step 6*, 100 μ l of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 μ l 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 mCAR assay kit includes a 10 mM stock solution of **TCPOBOP**, a common reference agonist of mCAR. The following 7-point treatment series, with concentrations presented in 4-fold decrements, provides a suitable dose-response: 12000, 3000, 750, 188, 46.9, 11.7, and 2.93 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. Perform a *rapid thaw* of the frozen cells by transferring a 10 ml volume of 37°C CRM into the tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 12 ml.

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

5.) 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 μ l of cell suspension into each well of the 96-well Assay 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. 7). Dispense 100 μ l of cell suspension into each well of the 96-well Assay Plate.

NOTE 5.1: Take special care to prevent cells from settling during the dispensing period. Allowing cells to settle during the transfer process, and/or lack of precision in dispensing uniform volumes across the assay plate *will* cause well-to-well variation (= increased Standard Deviation) in the assay.

NOTE 5.2: Users sometimes prefer to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed (100 μ l/well) into a clear 96-well cell culture treated assay plate, followed by the addition of 100 μ l/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 μ l of 2x-concentration treatment media into appropriate assay wells.

7.) Transfer the assay plate into a 37°C, humidified 5% CO₂ incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity ($\geq 85\%$) 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 mCAR activity, remove **Detection Substrate** from the refrigerator and place them in a low-light area so that it may equilibrate to room temperature. Gently invert the tube several times to ensure an homogenous solution.

NOTE: Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.

10.) Set the plate-reader to "luminescence" mode. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, *or less*.

11.) *Immediately before proceeding to Step 12*, 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.) Dispense 100 µl per well of **LDR** into 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.

14.) Quantify luminescence.

V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
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 Kit 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
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
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 in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats
LCM-10	Reagent in 10x-bulk volume to perform 960 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats

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, animals, or plants.

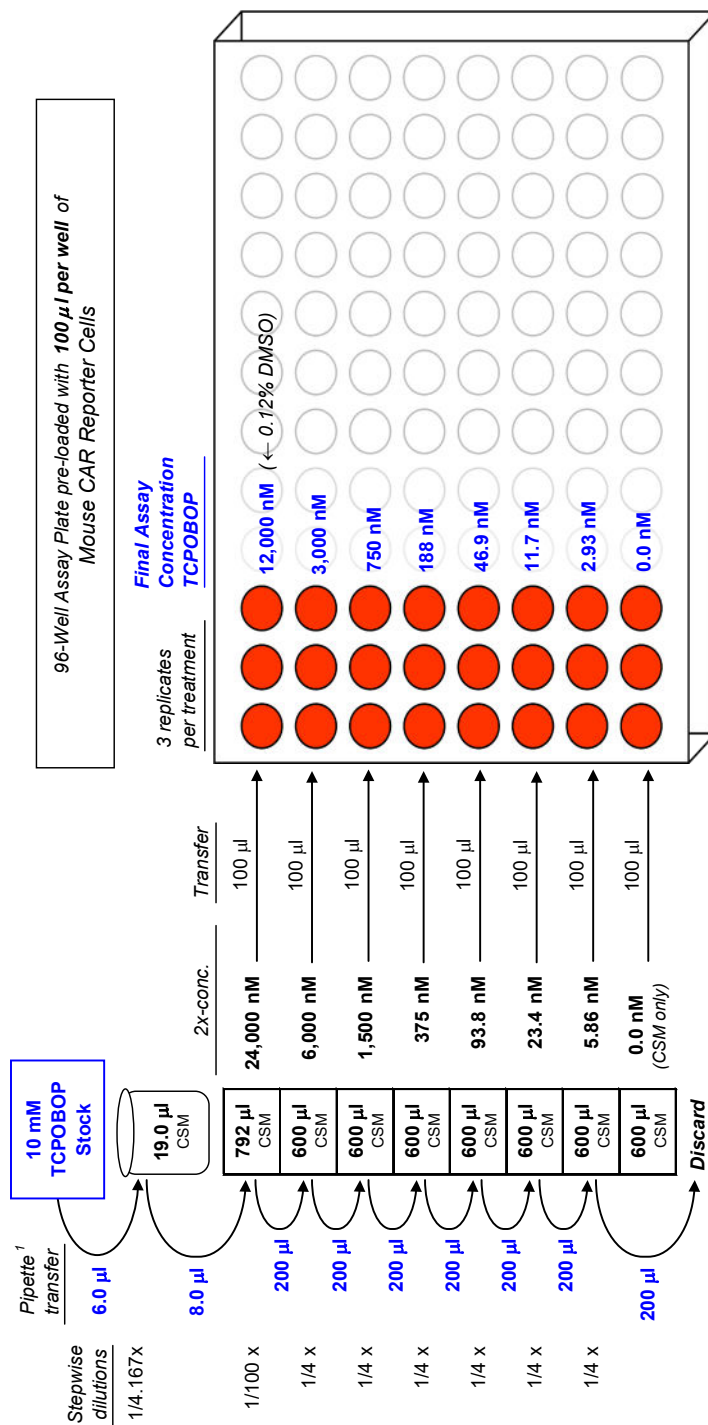
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APPENDIX 1

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



¹ For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.