Human Mineralocorticoid Receptor
(NR3C2, MR)
Reporter Assay System

3x32 Assays in 96-well Plate Format
Product # IB00501-32

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Technical Manual
(Generation 3 version 7.2)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

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

I. Description

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▪ The Assay System ▪

This nuclear receptor assay utilizes proprietary cells engineered to provide constitutive high-level expression of full-length, unmodified **Human Mineralocorticoid Receptor (NR3C2),** a ligand-dependent transcription factor referred to as **MR**.

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to a MR-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in MR activity. Luciferase gene expression occurs after ligand-bound MR undergoes nuclear translocation, DNA binding, recruitment and assembly of the co-activators and accessory factors required to form a functional transcription complex, culminating in expression of the reporter gene. Unlike some other cell-based assay strategies, the readout from INDIGO's reporter cells demands the same orchestration of all intracellular molecular interactions and events that can be expected to occur *in vivo*.

MR 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 cumbersome intermediate treatment steps such as spin-and-rinse of cells, viability determinations, or cell titer adjustments prior to assay setup.

INDIGO Bioscience's Reporter Assays are all-inclusive cell-based assay systems. In addition to MR Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, the reference agonist Aldosterone, Luciferase Detection Reagent, and a cell culture-ready assay plate.

▪ The Assay Chemistry ▪

INDIGO's cell-based 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 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 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 ▪

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 Aldosterone, **Compound Screening Medium (CSM)** is used as the diluent to make serial dilutions of test compounds to achieve the desired assay concentrations.

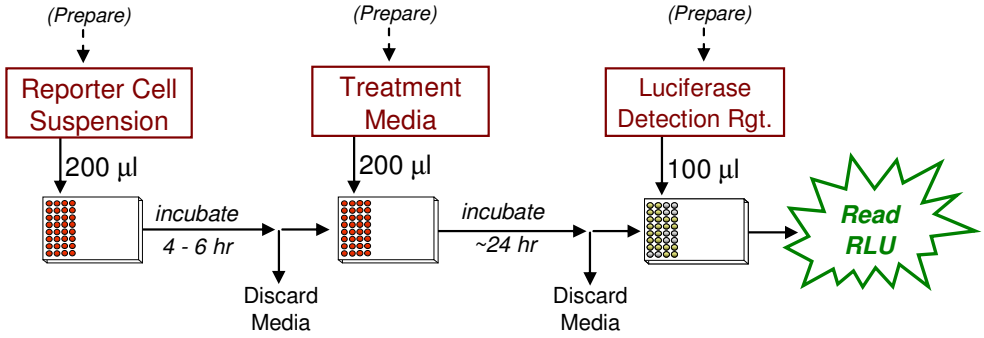
Alternatively, if test compound solubility is expected to be problematic,
2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared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

Regardless of the dilution method used, the concentration of total DMSO (or any organic solvent) 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.



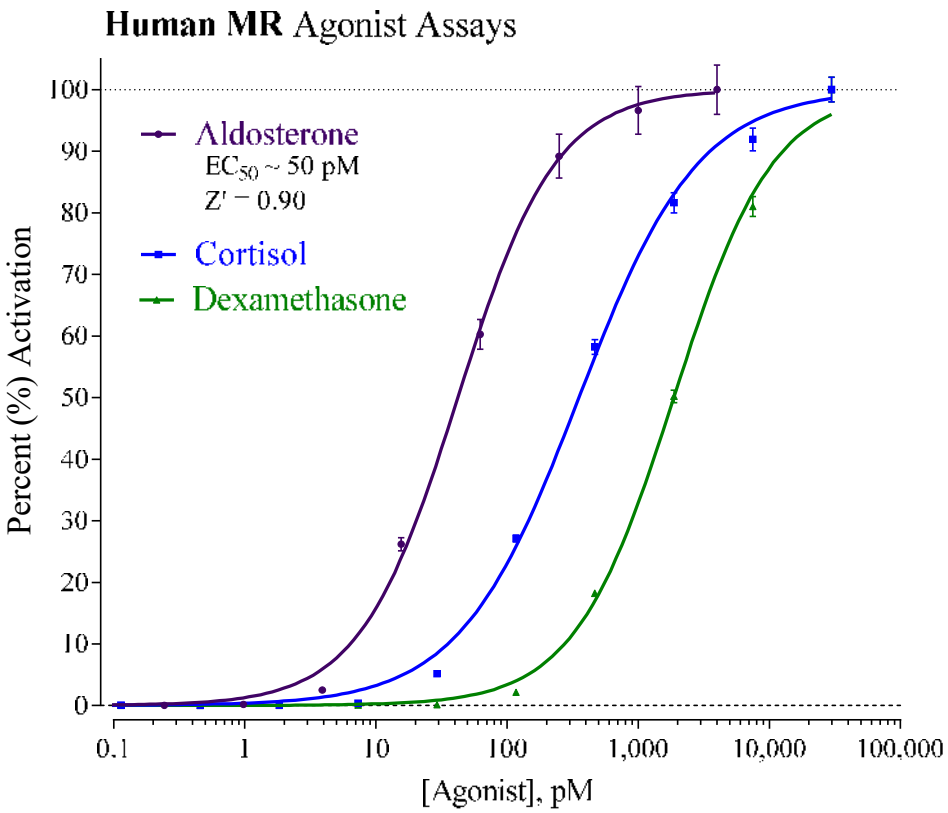


Figure 2. Agonist dose-response analyses of the Human MR.
Analyses of MR Reporter Cells using Aldosterone (provided), Cortisol and Dexamethasone (Cayman Chemical). Concentrated stocks of agonists were prepared in DMSO, then serially diluted using CSM. Final assay concentrations for each agonist were: 4000, 1000, 250, 62.5, 15.6, 3.91, 0.977 and 0 pM. Luminescence was quantified and average relative light units (RLU) and corresponding standard deviation (SD), % coefficient of variation (%CV), and Relative Percent Activation were determined for each treatment concentration (n = 4). Z' values were calculated as described by Zhang, *et al*. (1999)[1]. GraphPad Prism software was used to perform the least squares method of non-linear regression and to determine EC_{50} values.

The low %CV and high Z' score confirm the robust performance of this MR Assay and its suitability for HTS Applications.

[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.

$$Z' = 1 - [3*(SD^{Ref.\ EC100} + SD^{Bkg.}) / (RLU^{Ref.\ EC100} - RLU^{Bkg})]$$

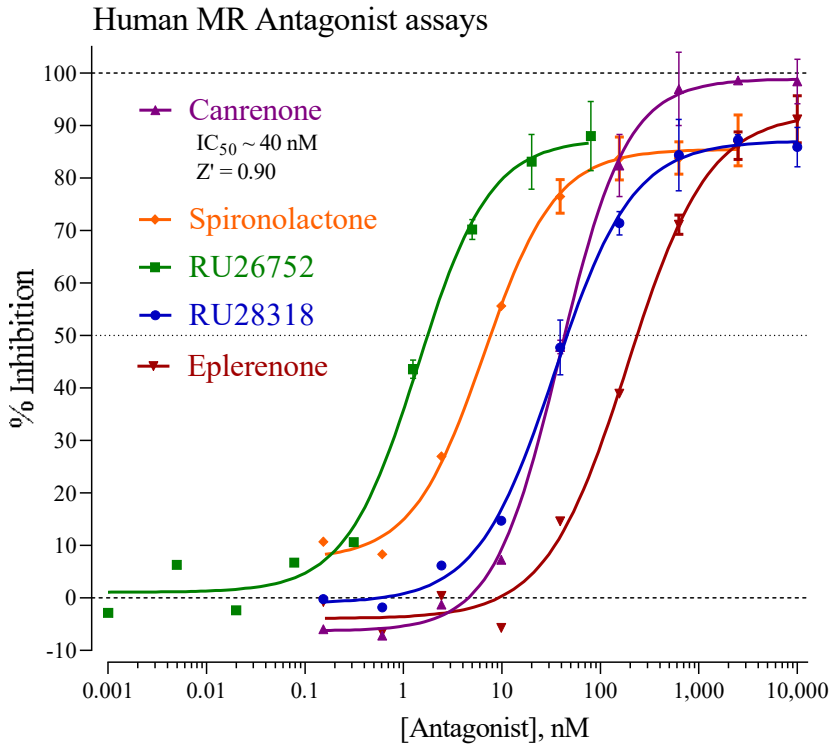


Figure 3. Validation of *Antagonist*-mode MR Assays

Human MR antagonist assay performance was validated using the reference antagonists Canrenone (Cayman Chemical), Spironolactone, RU26752, RU28318 and Eplerenone (all from Tocris). Assay setup and quantification of MR activity were performed following the protocol described in this Technical Manual. Human MR Reporter cells were co-treated with a fixed ~EC_{80} concentration of Aldosterone (140 pM) and varying concentrations of the respective antagonist compounds. Assay concentrations of Canrenone, Spironolactone, RU28318 and Eplerenone ranged between 10 µM and 150 pM. Treatment concentrations of RU26752 ranged from 80 nM to 10 pM. 'No antagonist' controls were included. Assay plates were incubated for 22 hrs, then processed to quantify MR activity for each treatment condition.

II. Product Components & Storage Conditions

This Human MR 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 Reporter 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 tubes 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.*
▪ MR 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
▪ Aldosterone, 4.0 µM (in DMSO) (reference agonist for MR)	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:* reference antagonist (refer to Figure 3)
▪ *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 actual bench work plus a 4-hour pre-incubation step. *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 MR Assay kit includes a 4.0 µM stock solution of **Aldosterone**, a potent activator of MR that may be used to setup antagonist-mode assays. 140 pM Aldosterone typically approximates EC_{80} in this reporter assay. Hence, it provides a suitable assay concentration of agonist to be used when screening test compounds for inhibitory activity to MR.

Add the challenge agonist (Aldosterone) 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 'cell blank' wells (meaning cell-free but containing 'Compound Screening Media') must be included in the assay plate to allow quantification of plate-specific 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, preferably collagen-coated, 96-well assay plate. Continue to process this 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 assay kit includes a 4.0 µM stock solution of **Aldosterone**, a potent activator of MR. The following 7-point treatment series, prepared in serial 4-fold decrements, provides a complete dose-response: 4000, 1000, 250, 62.5, 15.6, 3.91, and 0.977 pM. Always include a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

~ or ~

***b. Antagonist*-mode assays**. When setting antagonist assays, first supplement a bulk volume of CSM with the challenge agonist (Aldosterone) to achieve the desired fixed assay-concentration (refer to "*A word about antagonist-mode assay setup*", pg. 8). The agonist-supplemented CSM is then used to make dilutions of test compound stock(s) 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. Set the read time to 0.5 second (500 mSec) per well, *or less*.

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

Product No.	*Product Descriptions*
Human MR Assay Kit Products	
IB00501-32	Human MR Reporter Assay System 3x 32 assays in 96-well format
IB00501	Human MR Reporter Assay System 1x 96-well format assay
IB00502	Human MR Reporter Assay System 1x 384-well format assays
Alternative volumes of Bulk Assay Reagents may be custom manufactured to better meet customer's needs. Please Inquire.	

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

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

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VI. Limited Use Disclosures

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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 recnently updated version.

APPENDIX 1

Example scheme for the serial dilution of Aldosterone reference agonist using CSM, and the setup of a Human MR dose-response assay.