

**Human Mineralocorticoid Receptor
(NR3C2, MR)
Reporter Assay System**

96-well Format Assays
Product # IB00501

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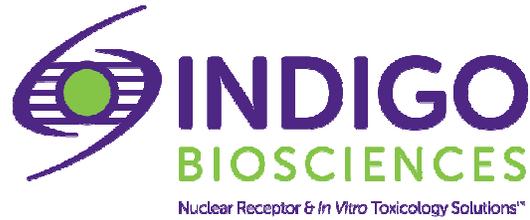
Technical Manual
(version 7.1)

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Human MR Reporter Assay System 96-well Format Assays

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I. Description

▪ The Assay System ▪

This nuclear receptor assay system utilizes proprietary non-human cells engineered to provide constitutive high-level expression of full-length, unmodified **Human Mineralocorticoid Receptor (NR3C2)**, a ligand-dependent transcription factor commonly 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 exceptional 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, cell titer adjustments, or the pre-incubation of reporter cells prior to assay setup.

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

▪ Preparation of Test Compounds ▪

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using **Compound Screening Medium (CSM)**, as described in *Step 2* of the **Assay Protocol**. This method avoids the adverse effects of introducing high concentrations of DMSO into the assay. The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

NOTE: CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup, and are considered to be 'single-use' reagents.

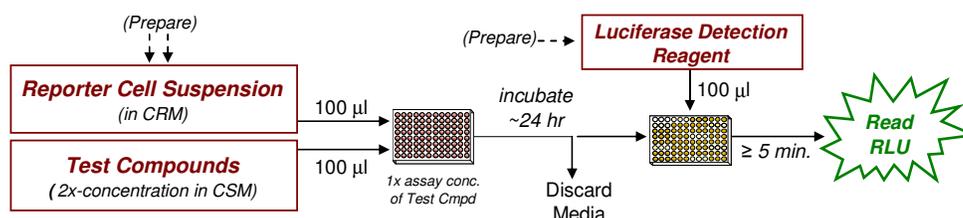
▪ Considerations for Automated Dispensing ▪

When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

Stock Reagent & Volume provided	Volume to be Dispensed (96-well plate)	Excess rgt. volume available for instrument dead volume
Reporter Cell Suspension 12 ml <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 ▪

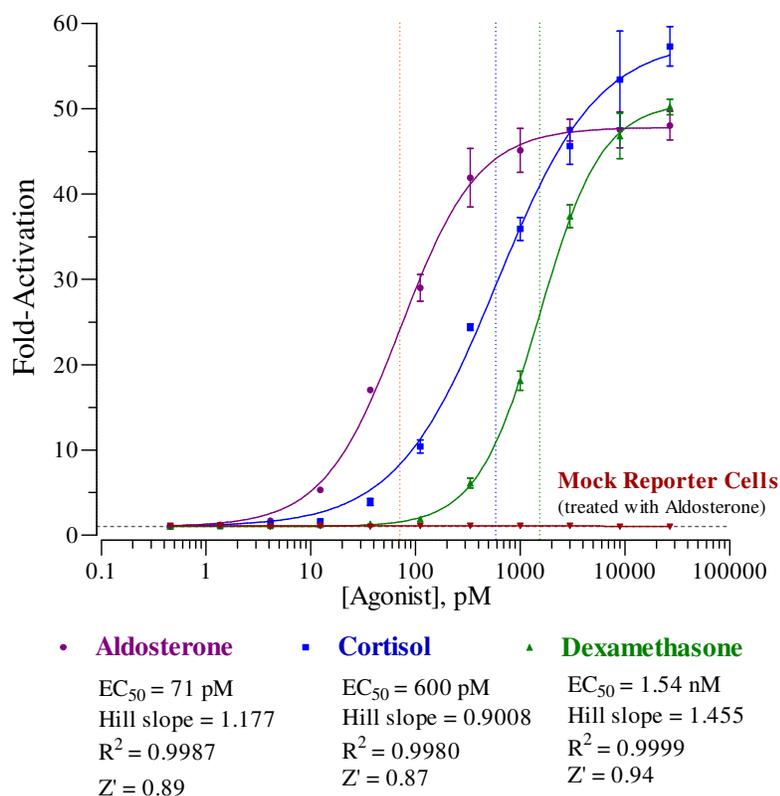


Figure 2. Agonist dose-response analyses of the Human MR assay.

Analyses of MR Reporter Cells using Aldosterone (provided), Cortisol (Tocris), and Dexamethasone (Cayman Chemical). In addition, to assess the level of background signal contributed by non-specific factors that may cause activation of the luciferase reporter gene, “mock” reporter cells, which contain only the luciferase vector, were treated with Aldosterone (mock reporter cells are not provided with assay kits). Concentrated stocks of agonists were prepared in DMSO, then serially diluted using CSM. Final assay concentrations for each agonist were: 27000, 9000, 3000, 1000, 333, 111, 37.0, 12.3, 4.12, 1.37, 0.457 and 0 pM. The highest assay concentration of DMSO was ≤ 0.07%. Luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 6). Signal-to-background (S/B) 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.

Mock reporter cells demonstrate no significant background, thus, luminescence results strictly through ligand-activation of Human MR expressed in these reporter cells. The low %CV, high S/B and exceptional Z' scores 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.

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

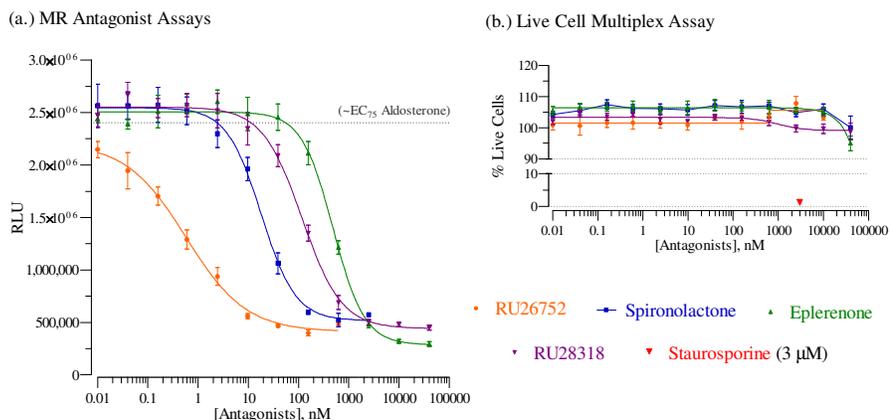


Figure 3. Validation of MR Assay *antagonist* dose-responses (a) performed in combination with INDIGO's Live Cell Multiplex Assay (b).

MR Antagonist assays were performed using RU26752, Spironolactone, Eplerenone, and RU28318 (all from Tocris). Assay setup and quantification of MR activity were performed following the protocol described in this Technical Manual. To confirm that the observed drop in RLU values resulted from receptor inhibition, not induced cell death, the relative numbers of live cells in each assay well were determined at the end of the treatment periods using INDIGO's Live Cell Multiplex (LCM) Assay (#LCM-01).

Final assay concentrations of the respective antagonists ranged between 40 μM and 10 pM , and included a 'no antagonist' control ($n \geq 6$ per treatment; highest [DMSO] $\leq 0.25\%$ *f.c.*). Each treatment also contained 110 pM ($\sim \text{EC}_{75}$) of the agonist Aldosterone. In addition, a set of assay wells were treated with 3 μM Staurosporine, thereby providing the LCM assay a positive control for 'cell death'. Assay plates were incubated for 23 hrs, then processed according to the LCM Assay protocol to quantify relative numbers of live cells per treatment condition. The assay plate was then further processed to quantify MR activity for each treatment condition.

Results: LCM Assays confirmed that the MR Reporter Cells experienced no significant variance in the numbers of live cells per assay well between treatment concentrations for all four antagonists. Hence, the observed dose-dependent reductions in RLU values are due to receptor inhibition, and *not* treatment-induced cell death.

II. Product Components & Storage Conditions

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

The aliquot of MR 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><i>Kit Components</i></u>	<u><i>Amount</i></u>	<u><i>Storage Temp.</i></u>
▪ MR 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
▪ Aldosterone, 10 µM (in DMSO) (reference agonist for MR)	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

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 9-14* 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 known agonist AND the test compound(s) to be evaluated for antagonist activity. This MR Reporter Assay System kit includes a 10 µM stock solution of **Aldosterone**, a potent agonist of MR that may be used to setup antagonist-mode assays. 333 picoMolar (pM; 10⁻¹² Molar) Aldosterone typically approximates EC₇₅ in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

We find that adding the reference agonist to the bulk suspension of Reporter Cells (*i.e.*, prior to dispensing into assay wells) is the most efficient and precise method of setting up antagonist assays, and it is the method presented in *Step 5b* of the following protocol. Note that, in *Step 6*, 100 µl of treatment media is combined with 100 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.

DAY 1 Assay Protocol: All steps must be performed using 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 (first see *Note 5.3*): Prepare Test Compound treatment media for *Agonist-* or *Antagonist-mode* screens.

The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

Note that, in *Step 6*, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl of Reporter Cells. Hence, to achieve the desired *final* assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Use **CSM** to prepare the appropriate dilution series. Manage dilution volumes carefully. This assay kit provides 35 ml of CSM.

Preparing the positive control: This MR Reporter Assay System kit includes a 10 µM stock solution of **Aldosterone**, a reference agonist of MR. The following 8-point treatment series, with concentrations presented in 5-fold decrements, provides a suitable dose-response: 3000, 1000, 333, 111, 37.0, 12.3, 4.12, and 1.37 pM (final assay concentrations), and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

3.) Rapid Thaw of the Reporter Cells: *First*, retrieve the tube of **CRM** from the 37°C water bath and sanitize the outside surface with a 70% ethanol swab.

Second, retrieve **Reporter Cells** from -80°C storage and, *without delay*, perform a rapid thaw of the frozen cells by transferring a 10 ml volume of the pre-warmed CRM into the tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 12 ml.

4.) Retrieve the tube of Reporter Cell Suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab, then transfer it into the cell culture hood.

5.) **a. Agonist-mode assays.** Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into each well of the 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. 8). Dispense 100 µl of cell suspension into each well of the 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 wish to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed (100 µl/well) into a clear 96-well cell culture treated assay plate, followed by 100 µl/well of CSM. Incubated overnight in identical manner to those reporter cells contained in the white assay plate.

NOTE 5.3: For logistical reasons, some users find it more convenient to first plate the reporter cells and then prepare their test compound dilutions. That strategy works equally well. Once plated, cells may be placed in an incubator for up to 3 hours before proceeding to *Step 6*.

6.) Dispense 100 µl 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 (≥ 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 MR activity, remove **Detection Substrate** and **Detection Buffer** from the refrigerator and place them in a low-light area so that they may equilibrate to room temperature. Once at room temperature, gently invert each tube several times to ensure homogenous solutions.

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

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

11.) *Immediately before proceeding to Step 12*, transfer the entire volume of Detection Buffer into the vial of Detection Substrate, thereby generating a 12 ml volume of **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

12.) Following 22 - 24 hours of incubation discard all media contents by ejecting it into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets. Cells will remain tightly adhered to well bottoms.

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

14.) Quantify luminescence.

V. Related Products

MR Assay Products	
<i>Product No.</i>	<i>Product Descriptions</i>
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 needs. Please Inquire.	

LIVE Cell Multiplex (LCM) Assay	
<i>Product No.</i>	<i>Product Descriptions</i>
LCM-01	Reagent volumes sufficient to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well NR assay plates
LCM-05	Reagents in 5x-bulk volume to perform 480 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well NR assay plates
LCM-10	Reagents in 10x-bulk volume to perform 960 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well NR assay plates

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Limited Use Disclosures

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APPENDIX 1

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

