

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

96-well Format Assays
Product # IB00501

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Technical Manual
(Generation 3 version 7.2)

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Human MR Reporter Assay System 96-well Format Assays

I. Description	
▪ The Assay System.....	3
▪ The Assay Chemistry.....	3
▪ Preparation of Test Compounds.....	4
▪ Considerations for Automated Dispensing.....	4
▪ Assay Scheme.....	4
▪ Assay Performance.....	5
II. Product Components & Storage Conditions	7
III. Materials to be Supplied by the User	7
IV. Assay Protocol	
▪ A word about <i>Antagonist</i> -mode assay setup.....	8
▪ <i>DAY 1 Assay Protocol</i>	8
▪ <i>DAY 2 Assay Protocol</i>	10
V. Related Products	11
VI. Limited Use Disclosures	11
APPENDIX 1: Example Scheme for Serial Dilutions	12

I. Description

▪ The Assay System ▪

This nuclear receptor assay system 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 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 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)** may be used as the diluent to make serial dilutions of test compounds to achieve the desired assay concentration series.

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%. Significant 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 considered to be 'single-use' reagents.

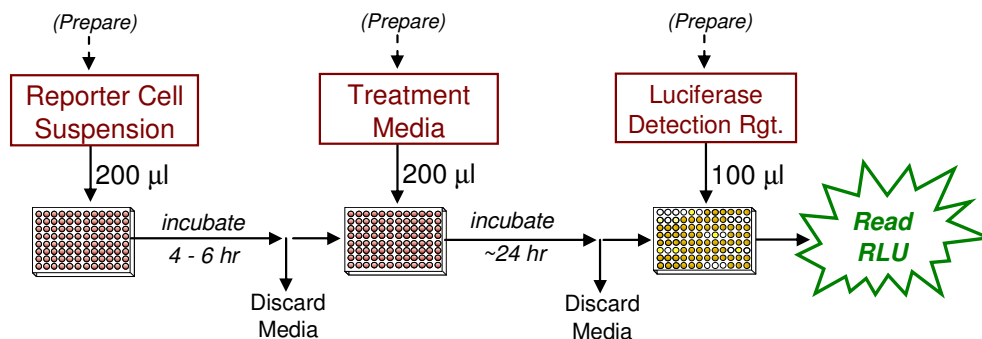
▪ Considerations for Automated Dispensing ▪

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 plumbing; 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 1x-concentration 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.



Human MR Agonist Assays

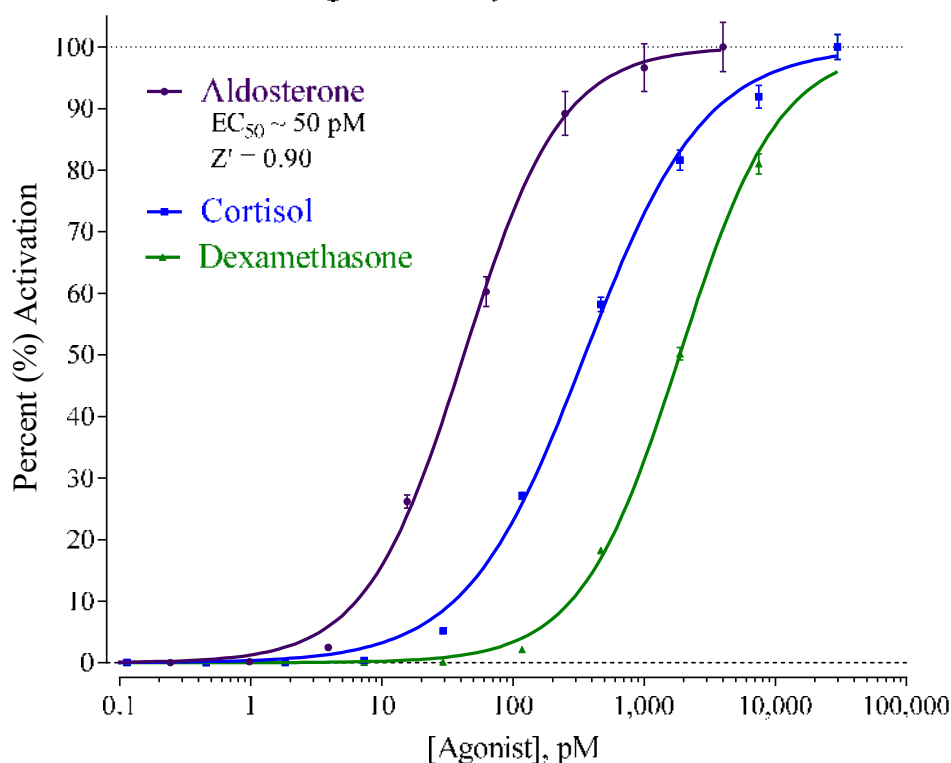


Figure 2. Agonist dose-response analyses of the Human MR.

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 EC₅₀ 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.

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

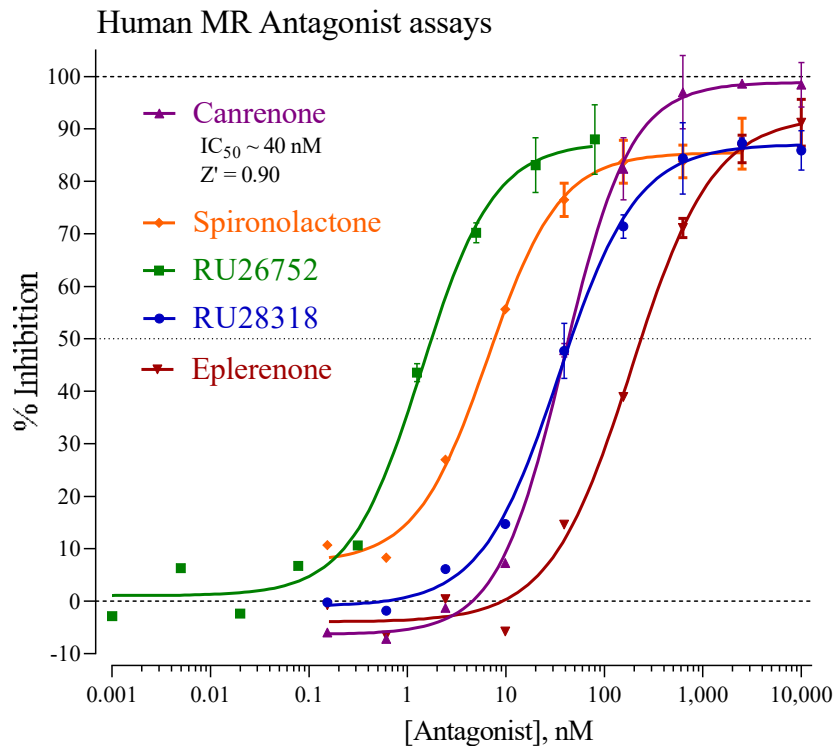


Figure 3. Validation of Antagonist-mode MR Assays

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 $\sim EC_{80}$ concentration of Aldosterone (140 pM) and varying concentrations of the respective antagonist compounds. 'No antagonist' controls were included. Assay plates were incubated for 22 hours, then processed to quantify MR activity for each treatment condition.

II. Product Components & Storage Conditions

This Human MR Assay kit contains materials to perform assays in a single 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 3 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ MR 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
▪ Aldosterone, 4.0 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, <i>collagen-coated</i> 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 -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% CO₂ incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media basins, sterile.
- sterile 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 Fig. 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 EC₅₀ – EC₈₅) 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 EC₈₀ 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 EC₅₀ – EC₈₅ 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-channel pipette, dispense **200 μl / well** of cell suspension into the 96-well 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. 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% CO₂) 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 at the desired assay concentrations:** Use CSM to prepare an appropriate dilution series of the reference and test compound stocks. Prepare treatment media at the desired **assay concentrations**. In *Step 9*, **200 µl / well** of the prepared treatment media are dispensed 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 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 assay-concentrations (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 4-6 hours pre-culture period, discard the media. The preferred method is to use a 'wrist flick' to manually 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 each prepared treatment media into the assay plate.

Hint: 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-channel 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 cell culture 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 is set to 0.5 second (500 mSec) per well, *or less*.

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 assay plate to rest at room temperature for 5 - 10 minutes following the addition of LDR. Do not shake the assay plate during this period.

17.) Quantify luminescence.

V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
Human Mineralocorticoid Receptor (MR) Assay Kit Products	
IB00501-32	Human MR Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB00501	Human MR Reporter Assay System 1x 96-well format assay
IB00502	Human MR Reporter Assay System 1x 384-well format assays
Mouse Mineralocorticoid Receptor (MR) Assay Kit Products	
M00501	Mouse MR Reporter Assay System 1x 96-well format assay
Alternative volumes of Bulk Assay Reagents may be custom manufactured to meet customers' 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

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 TM 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 recently updated version.

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APPENDIX 1

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

