Human Mineralocorticoid Receptor
(NR3C2, MR)
Reporter Assay System

384-well Format Assays
Product # IB00502

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Technical Manual
(Generation 3_version 8.0)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

Human MR Reporter Assay System
384-well Format Assays

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 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 Assays feature a luciferase detection reagent specially formulated to provide stable light emission between 30 and 100+ minutes after initiating the luciferase reaction. Incorporating a 30-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.

▪ Considerations for the Preparation and Automated Dispensing of Test compounds ▪

Small molecule compounds are typically solvated at high concentration (ideally 1,000x-concentrated) in DMSO and stored frozen as master stocks. For **384-well format assays** the user will choose to dilute master stocks using one of two alternative methods. The selection of dispensing method to be used will be dictated by the type of instrument that will be used. This Technical Manual provides detailed protocols for each of these two alternative methods:

a.) Assay setups in which a conventional **tip-based** instrument is used to dispense **µL volumes** of test compounds into assay wells (protocol is presented in black text). Use **Compound Screening Medium (CSM)** to generate a series of **2x-concentration** test compound treatment media, as described in *Step 2a* of the **Assay Protocol**. The final concentration of DMSO carried over into assay reactions should never exceed 0.4%; strive to use 1,000x-concentrated stocks when they are prepared in DMSO.

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.

and,

b.) **Acoustic transfer** or **Pin-based dispensing of nL volumes** of test compounds into assay wells (protocol is presented in blue text). Use DMSO to make a series of **1,000x-concentrated** test compound stocks that correspond to each desired final assay concentrations, as described in *Step 2b* of the **Assay Protocol**.

▪ Considerations for Automated Dispensing of Other Assay Reagents ▪

When dispensing into a small number of assay plates, first carefully consider the dead volume requirement of your tip-based 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 on a *per kit* basis. Always pool the individual reporter cell suspensions and all other respective assay kit reagents before processing multiple 384-well assay plates.

Stock Reagent & Volume provided	**Volume to be Dispensed** *(384-well plate)*	**Excess rgt. volume available for instrument dead volume**
when using tip dispensing of test cmpds **Reporter Cell Suspension** 7.5 mL	15 µL / well 5.8 mL / plate	~ 1.7 mL
when using acoustic dispensing of test cmpds **Reporter Cell Suspension** 15 mL	30 µL / well 11.5 mL / plate	~ 3.4 mL
Detection Substrate 7.8 mL	15 µL / well 5.8 mL / plate	~ 2 mL

▪ Assay Scheme ▪

The *Day 1* preparation, volumes, and chronology of dispensed cells and test compounds are different between assay setups using a *tip-based dispenser* (**1a**) and those using an *acoustic transfer device* (**1b**). Following ~ 24 hours incubation of treated Reporter Cells, Detection Substrate is added. Light emission from each assay well is quantified using a plate-reading luminometer.

Figure 1a. Assay workflow if using conventional **tip-based** dispensing of test compounds.

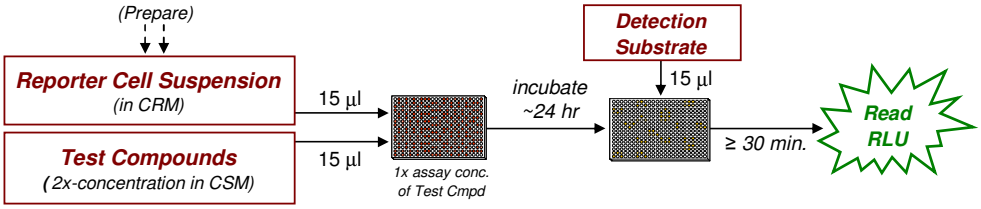
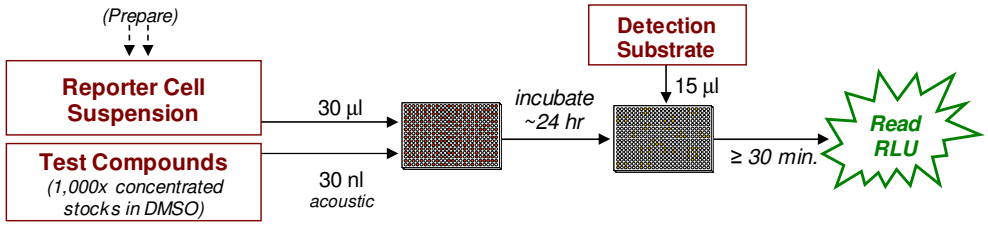


Figure 1b. Assay workflow if using **acoustic** dispensing of test compounds.



▪ Assay Performance ▪

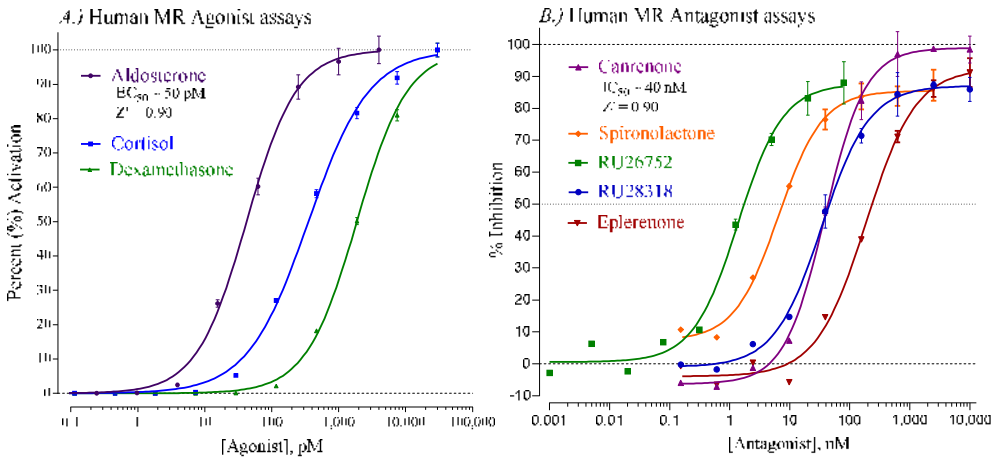


Figure 2. Agonist and Antagonist dose-response analyses of Human MR.

A.) Agonist analyses of MR Reporter Cells using Aldosterone (provided), Cortisol and Dexamethasone (Cayman Chemical). *B.)* Antagonist assay performance was validated using the reference antagonists Canrenone (Cayman Chemical), Spironolactone, RU26752, RU28318 and Eplerenone (all from Tocris). Concentrated stocks of reference compounds were prepared in DMSO, then serially diluted using CSM. Luminescence was quantified and average relative light units (RLU) and corresponding standard deviation (SD), and % coefficient of variation (%CV) were determined for each treatment concentration (n = 4). Z' values were calculated as per Zhang, *et al.* (1999)[1]. All treatment concentrations were Log10 transformed. Agonist responses were normalized in terms of Fold-Activation, whereas antagonist responses are plotted in terms of % Inhibition. Data were plotted using the least-squares method of non-linear regression and EC_{50} / IC_{50} values were determined using GraphPad Prism.

[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.

II. Product Components & Storage Conditions

This Human MR Assay kit contains materials to perform assays in a single 384-well assay plate.

Cryopreserved mammalian 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 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 inventory the individual kit components be sure to first transfer and submerge the tube of cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, the 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	1 x 1.0 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	1 x 7.0 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 80 µL	-20°C
▪ Detection Substrate	1 x 7.8 mL	**-80°C**
▪ 384-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

▪ dry ice container

▪ 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 & tips suitable for dispensing 15 µL.

▪ disposable media basins, sterile.

▪ sterile multi-channel media basins *or* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).

▪ *Optional:* antagonist reference compound (*e.g.,* Fig. 2B)

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 9-13* 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 varying concentrations of the test compound(s) to be evaluated for antagonist activity. 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 assay. Hence, it provides a suitable final assay concentration of agonist to be used when screening test compounds for inhibitory activity to MR.

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 protocol when performing tip-based dispensing, and *Step 6b* of the protocol when using an acoustic transfer device to dispense test compounds.

Note that when using a *tip-based instrument* for the dispensing of 2x-concentrated test compounds the cell suspension must also be supplemented with a **2x-**concentration of the challenge agonist aldosterone (~280 pM).

When using an *acoustic transfer* device for the dispensing of 1,000x-concentrated test compounds the cell suspension should be supplemented with a **1x-**concentration of the challenge agonist aldosterone (~140 pM).

DAY 1 Assay Protocol:

All steps must be performed using proper 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. NOTE that test and reference compounds will be prepared differently when using tip-dispensing *vs.* acoustic dispensing. Regardless of the method, the total DMSO carried over into assay reactions should never exceed 0.4%.

a. *Tip dispensing method*: In *Step 6*, 15 µL / well of the prepared treatment media is added to the assay that has been pre-dispensed with 15 µL /well 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. Plan dilution volumes carefully; this assay kit provides 45 mL of CSM.

b. *Acoustic dispensing method*: In *Step 6*, 30 nL / well of **1,000x**-concentrated test compound solutions (prepared in DMSO) are added to the assay plate using an acoustic transfer device.

Preparing the positive control: This assay kit includes a 4.0 µM stock solution of **Aldosterone**, a potent reference agonist 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 (final assay concentrations). Always include 'no treatment' control wells.

APPENDIX 1a provides an example for generating this dilution series to be used when *tip-dispensing* compound solutions prepared in CSM (15 µL / well).

APPENDIX 1b provides an example for generating a series of 1,000x-concentrated solutions of compounds prepared in DMSO to be used when performing *acoustic dispensing* (30 nL / well).

When using *tip-based* instrumentation for dispensing test compounds …

3.) *First*, retrieve the tube of **CRM** from the 37°C water bath, sanitize the outside with a 70% ethanol swab;

Second, retrieve **Reporter Cells** from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. Perform a *rapid thaw* of the frozen cells by transferring a **6.5 mL** volume of 37°C CRM into the tube of frozen cells. Recap the tube of Reporter Cells and place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.5 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.) Gently invert the tube of cell suspension several times to gain a homogenous suspension, then …

a. **for *Agonist*-mode assays**: Dispense **15 µL / well** of cell suspension into the Assay Plate.

~ or ~

b. **for *Antagonist*-mode assays**: Supplement the bulk volume of Reporter Cells suspension with a 2x-concentration of the challenge agonist (refer to "*A word about antagonist-mode assay setup*", pg. 7). Dispense **15 µL / well** of cell suspension into the Assay Plate.

6.) Dispense **15 µL / well** of 2x-concentrated treatment media (from *Step 2a*) into the assay plate.

When using an *acoustic transfer* device for dispensing test compounds …

3.) Dispense **30 nL / well** of the 1,000x-concentrated compounds (in DMSO solutions, from *Step 2b*) into the assay plate.

4.) *First*, retrieve the tube of **CRM** from the 37°C water bath, sanitize the outside with a 70% ethanol swab;

Second, retrieve **Reporter Cells** from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. Perform a *rapid thaw* of the frozen cells by transferring a **6.5 mL** volume of 37°C CRM into the tube of frozen cells. Recap the tube of cells and place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 7.5 mL.

5.) Retrieve the tube of cell suspension from the water bath. Sanitize the outside surface of the tube with a 70% alcohol swab. Add an additional **7.5 mL** of **CSM** to the tube. The resulting volume of cell suspension will be 15 mL.

6.) Gently invert the tube of cells several times to disperse cell aggregates and gain a homogenous cell suspension.

a. **for *Agonist*-mode assays**: Dispense **30 µL / well** of cell suspension into the Assay Plate that has been pre-dispensed with test compounds.

~ or ~

b. **for *Antagonist*-mode assays**: First supplement the bulk volume of MR Reporter Cells suspension with the challenge agonist **Aldosterone** to achieve an EC_{50} – EC_{80} concentration (refer to "*A word about antagonist-mode assay setup*", pg. 7). Then dispense **30 µL / well** of the supplemented cell suspension into the assay plate that has been pre-dispensed with test compounds.

NOTE: 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.

(continued …)

NOTE: Following the dispensing of Reporter Cells and test compounds INDIGO recommends performing a *low-speed* spin of the assay plate (with lid) for ≤ 1 minute using a room temperature centrifuge fitted with counter-balanced plate carriers.

7.) 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.

8.) For greater convenience on Day 2, retrieve **Detection Substrate** from freezer storage and place 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.) Approximately 30 minutes before intending to quantify receptor activity remove **Detection Substrate** from the refrigerator and place it in a low-light area so that it may equilibrate to room temperature. Gently invert the tube several times to ensure a homogenous solution.

NOTE: Do NOT actively warm Detection Substrate above room temperature. If this solution was 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.) Following 22 - 24 hours of incubation dispense **15 µL / well** of **Detection Substrate** to the assay plate.

NOTE: Perform this reagent transfer carefully to avoid bubble formation! Scattered micro-bubbles will not pose a problem. However, bubbles covering the surface of the reaction mix, or large bubbles clinging to the side walls of the well, will cause lens-effects that will degrade the accuracy and precision of the assay data. It is recommended to perform a final *low-speed* spin of the assay plate (with lid) for ≤ 1 minute using a room temperature centrifuge fitted with counter-balanced plate carriers.

12.) Allow the plate(s) to rest at room temperature for 30 minutes. Do not shake the assay plate(s) during this period.

NOTE: the 30-minute rest period allows the luminescence signal to achieve stable emission output.

13.) Quantify luminescence.

14.) Analyze data.

V. Related Products

Product No.	*Product Descriptions*
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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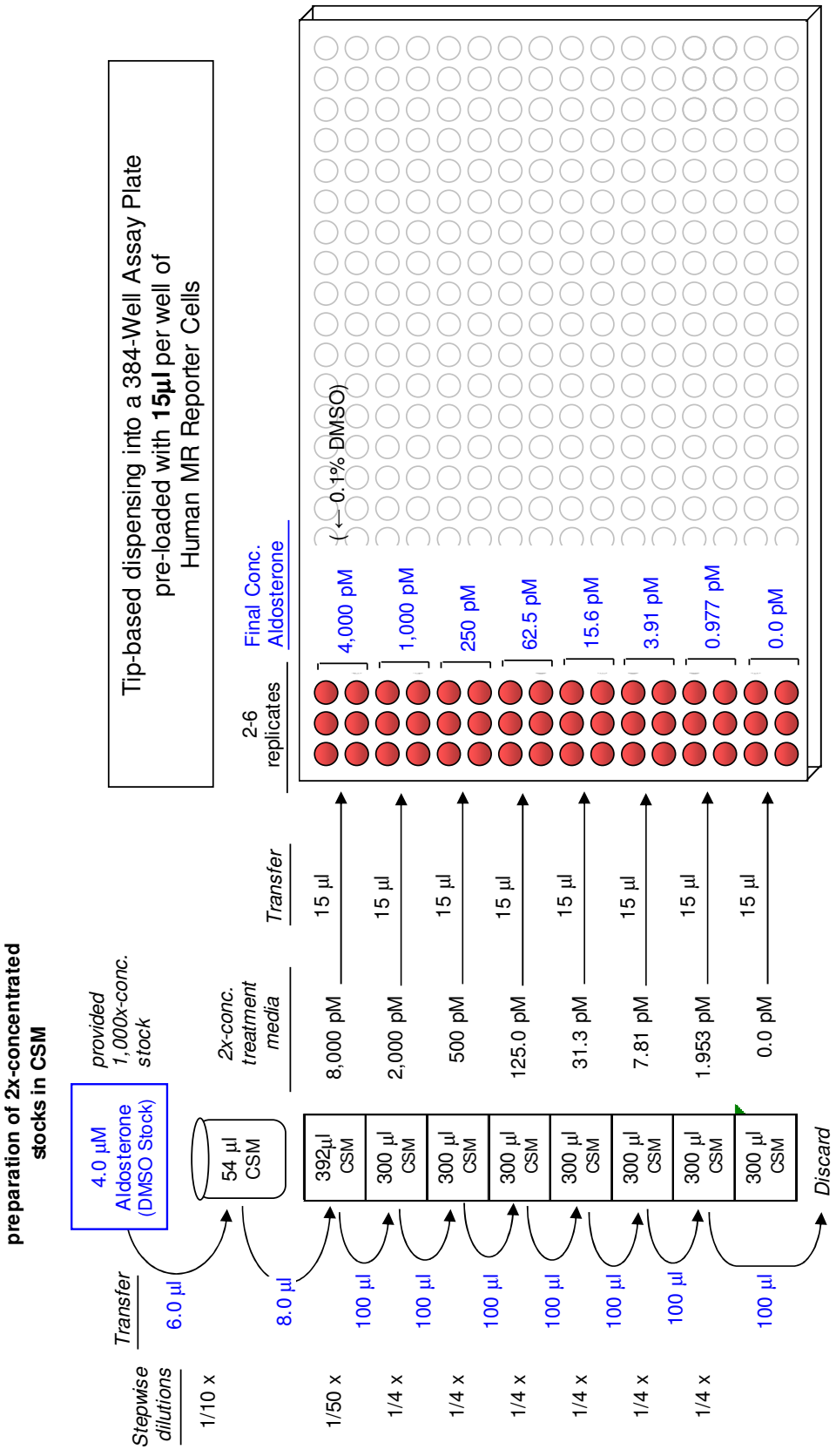
VI. Limited Use Disclosures

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 ™ 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.

APPENDIX 1a for tip-based dispensing. Example scheme for the serial dilution of the reference agonist Aldosterone into CSM to generate **2x-concentrated** treatment media. A *tip-based* instrument is used to dispense 15 µl / well into an assay plate that has been *pre-dispensed* with 15 µL / well of MR Reporter Cells suspension.



APPENDIX 1b for acoustic dispensing. Example scheme for the serial dilution of the reference agonist Aldosterone into DMSO to generate **1,000x-concentrated** stocks. 30 nL / well are pre-dispensed into an empty assay plate using an acoustic transfer device.