



INDIGO Biosciences, Inc.

The Nuclear Receptor Company™

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

96-well Format Assays
Product # IB00501

■

Technical Manual
(version 3.0)

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Human MR Reporter Assay System 96-well Format Assays

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I. Description

INDIGO Bioscience's Nuclear Receptor Reporter Assays are all-inclusive cell-based assay systems. In addition to human nuclear receptor reporter cells, kits provide an optimized cell culture medium, a medium for diluting test compounds, a positive-control agonist specific to each nuclear receptor, luciferase detection reagent, and a cell culture-ready assay plate. The primary application of these reporter assays is in the screening of test compounds to quantify functional activities, either agonist or antagonist, that they may exert against a specific human nuclear receptor.

These assay systems utilize reporter cells 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.

This **MR Reporter Assay System** utilizes non-human mammalian cells engineered to provide constitutive, high-level expression of **Human Mineralocorticoid Receptor (NR3C2)**, a ligand-dependent transcription factor commonly referred to as **MR**. Additionally, these cells contain an MR-responsive luciferase reporter gene. Thus, quantifying luciferase activity provides a surrogate measure of MR activity in the treated reporter cells.

INDIGO's nuclear receptor assays capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology. These nuclear receptor 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 oxyluciferin, AMP, PP_i , CO_2 , and photon emission. The luminescence intensity of the reaction is quantified using a plate-reading luminometer, and is reported in terms of relative light units (RLU).

In a typical assay setup, reporter cells are dispensed into wells of the assay plate and then immediately dosed with the user's test compounds. Following an overnight incubation, the treatment media are discarded and luciferase detection reagent (LDR) is added. The intensity of light emission from the ensuing luciferase reaction provides a sensitive measure that is directly proportional to the level of MR activation in the reporter cells.

All of INDIGO's Nuclear Receptor Reporter Assay Systems feature a luciferase detection reagent specially formulated to provide stable light emission between 15 and 90 minutes after initiating the luciferase reaction (refer to APPENDIX 2). There is no need to sequentially process-and-read single assay plates. The unwavering signal stability of this MR Nuclear Receptor Assay System enables batch processing of large numbers of assay plates, making this reporter chemistry optimally suited to meet the logistical challenges inherent to high-throughput screening (HTS).

II. Product Components & Storage Conditions

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

The individual aliquots of MR Reporter Cells and Detection Solutions I & II are provided as single-use reagents. Once thawed, reporter cells can NOT be refrozen with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay set-up.

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.

<i><u>Kit Components</u></i>	<i><u>Amount</u></i>	<i><u>Storage Temp.</u></i>
▪ MR Reporter Cells	1 x 2.0 mL	-80°C
▪ Cell Recovery Medium 1 (CRM-1)	1 x 10.5 mL	-20°C
▪ Compound Screening Medium 2 (CSM-2)	1 x 35 mL	-20°C
▪ Aldosterone, 10 µM (in DMSO) (control agonist for MR)	1 x 30 µL	-20°C
▪ Detection Solution I	1 x 6.0 mL	-80°C
▪ Detection Solution II	1 x 6.0 mL	-80°C
▪ 96-well assay plate (white, sterile, cell-culture ready)	1	ambient

III. Alternative Applications for this Reporter Assay

This nuclear receptor reporter assay system is a sensitive and versatile research tool. As such, the user may configure these assays in several ways to achieve different research objectives.

The “Assay Procedure” begins in Section V. It provides specific instructions for performing each MR assay, including alternative set-ups at Step 2 for the user to choose from. For example, these reporter assays may be configured to perform agonist dose-curves (Alternate 2A) or antagonist dose-curves (Alternate 2C), or to perform single-point screening of test compounds for agonist activities (Alternate 2B) or antagonist activities (Alternate 2D).

Due to the experiment-specific nature of these steps, these alternative procedures (and their attendant “NOTES”) are intended to serve as guidelines only. They are offered to assist researchers in formulating an assay design that is best suited to achieve their specific research goals.

Figure 1

Human MR Reporter Assay

Aldosterone Dose-Response, Normalized

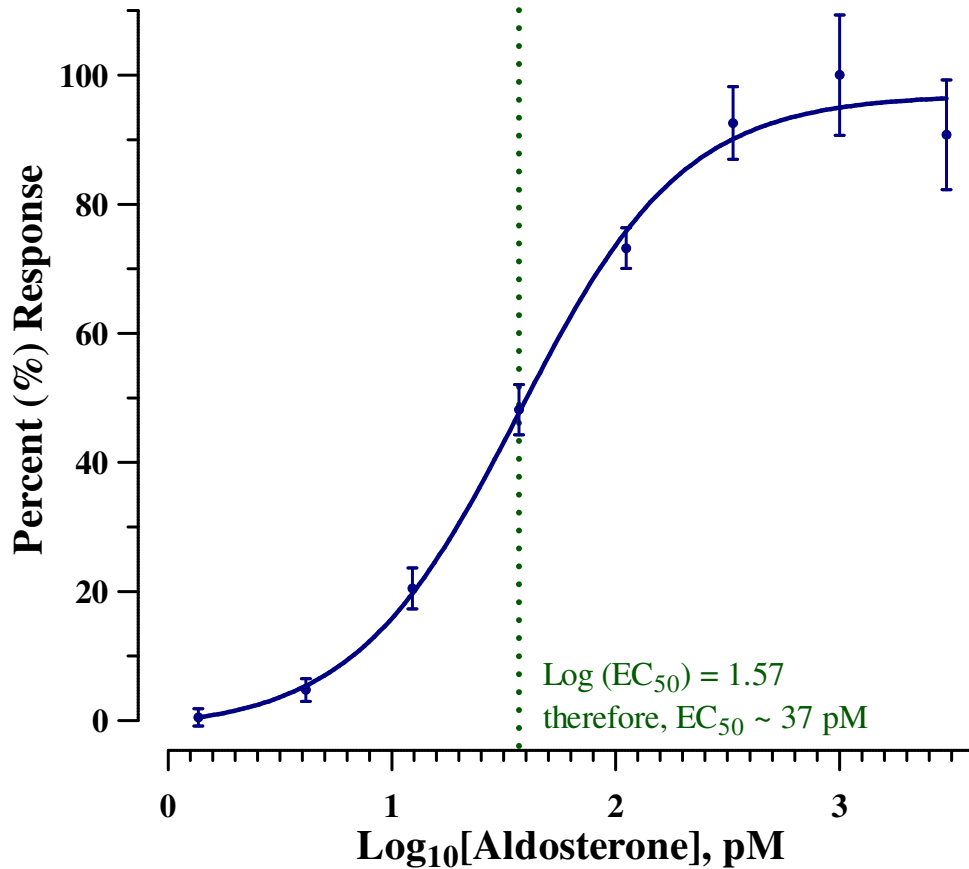


Figure 1. Representative agonist dose-response of the MR Reporter Assay System.

MR dose-response assays were performed according to the protocol provided in this Technical Manual. Reporter cells were treated with aldosterone at the following final concentrations: 3000, 1,000, 333, 111, 37.0, 12.3, 4.12, 1.37 and 0 pM. Treatment media were removed after 24 hr and LDR was applied directly to the cells. Luminescence was quantified using a Tecan GENios Pro plate-reading luminometer. Average relative light unit (RLU) and respective standard deviation values were determined. All numerical conversions and graphing were performed using GraphPad Prism software, as follows: Dose concentrations of aldosterone were transformed to = Log₁₀ (pM). RLU values were normalized such that the lowest RLU and the highest RLU values from each data set are defined as 0% and 100%, respectively. Non-linear regression was performed using "Log (agonist) vs. normalized response - Variable slope" analyses. Error bars depict %CV. The dotted line depicts a representative Log₁₀ (EC₅₀) concentration of aldosterone in this MR assay.

Figure 2

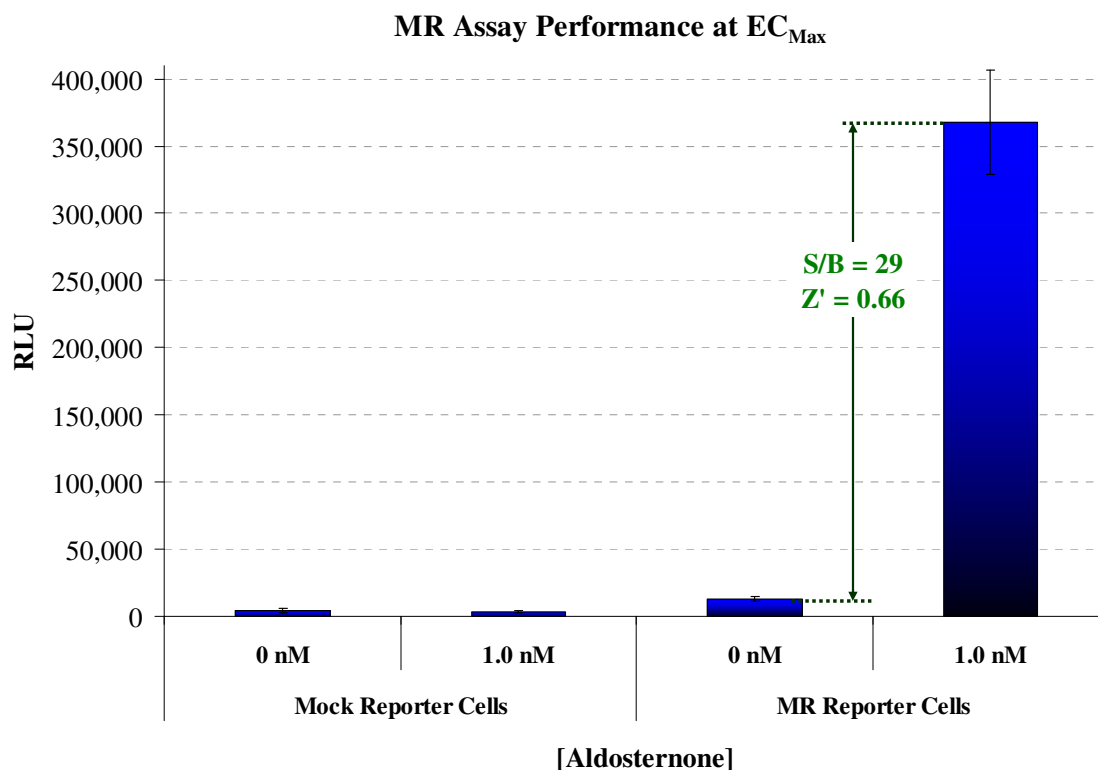


Figure 2. Validation of the MR reporter assay for screening applications.

MR reporter cells were treated with media alone, or media supplemented with 1 nM aldosterone. To assess the amount of background signal contributed by any other factor(s) that cause gratuitous activation of the luciferase reporter gene, “mock” reporter cells were specially prepared to contain only the luciferase vector (*i.e.*, cells withOUT the MR expression vectors. Mock reporter cells are not provided with assay kits). Mock reporter cells and the MR reporter cells were cultured, treated with aldosterone, and processed in identical manner. A minimum of six replicate assay wells were processed for each treatment condition. Luminescence was quantified as described in Figure 1. For the purposes of these analyses, RLU values are not background-subtracted. Signal-to-background (S/B) and Z' values were calculated as described by Zhang, *et al.* (1999)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.

RESULTS & CONCLUSIONS: In this particular experiment, MR reporter cells treated with 1 nM aldosterone yielded S/B = 29 and a calculated Z' value of 0.66. Similarly treated mock reporter cells demonstrate only low levels of background luciferase activity (< 1.1% of EC_{Max} treatment). Thus, luminescence results from ligand-activation of the exogenous human MR expressed in these reporter cells. These data confirm the robust performance of this MR Reporter Assay System, and demonstrate its suitability for use in HTS applications.¹

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.

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

IV. Materials to be Supplied by the User

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

DAY 1

- cell culture-rated hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath (*Step 3*).
- 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 control ligand(s) and user's test compound(s) (*Step 2*).
- *optional*: antagonist control compound (*Step 2*, Alternative Protocols C & D).
- 70% alcohol wipes (*Steps 3 & 4*).
- disposable media basin (*aka* 'media reservoir'), sterile (*Step 5*).
- 8- *or* 12-channel pipette & sterile tips appropriate for the transfer of 100 μ l volumes (*Steps 5 & 6*). The use of electronic pipettes capable of repeat-dispensing is recommended.

DAY 2

- plate-reading luminometer (*Step 10*).
- media basin and multi-channel pipette, as described above (*Step 13*).

V. Assay Procedure

Review the entire assay protocol before starting.

Completing the assay requires an overnight incubation. *Steps 1-8* are performed on **Day 1**, requiring 1 – 2 hours to complete. *Steps 9-15* are performed on **Day 2**, requiring \leq 1 hour to complete.

DAY 1: All steps must be performed using proper aseptic technique.

- 1) Remove **Cell Recovery Medium 1 (CRM-1)** and **Compound Screening Medium 2 (CSM-2)** from freezer storage and thaw.
- Room temperature **CSM-2** is used in the next step. A water bath may be used to facilitate rapid thawing of **CSM-2**.
 - Thaw **CRM-1** and warm to 37°C using a water bath. Pre-warmed **CRM-1** is required in *Step 3*.

ALTERNATIVE 2A: Agonist Dose-Response Assays (e.g., as depicted in Figure 1)

Use **CSM-2** to prepare a dilution series of 2x-concentrated control agonist (aldosterone) AND an appropriate dilution series of 2x-concentrated test compound(s) to be assayed.

NOTE 2.0 In *Step 6*, 100 µl of the prepared [CSM + test cmpd] is added into an assay well already containing 100 µl of Reporter Cells. Hence, to achieve the desired *final* concentration of test compound in the assay one must prepare and dispense a “2x-concentration” of the test compound(s) (*i.e.*, [CSM + 2x test cmpd]).

NOTE 2.1 When generating dose-response curves, it is recommended to perform all measurements in at least triplicate. In *Step 6*, 100 µl of [CSM + 2x test cmpd] will be added per well of the assay plate. Therefore, devise an appropriate compound dilution scheme to yield a final volume of [CSM + 2x test cmpd] that is slightly greater than 300 µl, thus allowing accurate volume transfers into each of the triplicate wells of the assay plate.

NOTE 2.2 When using aldosterone as the positive-control agonist, we find the following assay concentration range provides a complete dose-response: 3000, 1000, 333, 111, 37.0, 12.3, 4.12, 1.37 and 0 picoMolar (pM; 10^{-12} Molar), as depicted in **Figure 1**. However, as explained in *Note 2.0*, a 2x-concentrated dilution series of both the positive-control and test compounds are required.

[*Hint*: Generating this dilution series for aldosterone may be achieved by following the example presented in **APPENDIX 1**. In brief, use a portion of CSM-2 to first perform a 55.5-fold dilution of the provided 10 µM aldosterone stock (*e.g.*, mix 10 µl aldosterone stock with 545 µl of CSM). Use this intermediate stock to perform a subsequent 30-fold dilution to achieve the first desired 2x-concentration of 6000 pM aldosterone (containing 0.06% DMSO). Continue by using a portion of the 6000 pM solution to perform seven sequential 3-fold dilutions to produce 2000, 667, 222, 74.1, 24.7, 8.24 and 2.75 pM 2x-concentrated stocks. Neat CSM (or CSM supplemented with 0.06% DMSO; *i.e.*, the highest concentration of “vehicle”) may be used to provide the “0 nM” control treatment.]

ALTERNATIVE 2B: HTS of Compounds for Agonist Activities via Single-Point Assay

Use **CSM-2** to prepare a 2x-concentrated agonist "control" AND an appropriate 2x concentration of test compounds to be screened for agonist activity.

See *NOTE 2.0*.

This MR Reporter Assay System kit includes a 10 μ M stock solution of **Aldosterone**, a potent agonist of MR that may be used as a positive-control. An assay concentration of 1000 pM aldosterone typically provides $\geq 95\%$ activation of MR (Figure 1). Hence, 2000 pM aldosterone will provide a 2x-concentrated agonist that is a suitable positive-control reference. Refer to *Note 2.2* for a recommended dilution scheme to prepare the appropriate 2x-concentrated aldosterone solution.

NOTE 2.3 As a general rule, when an organic solvent is used to generate primary stock solutions of test compounds, we recommend that the user devise a dilution scheme so that the concentration of organic solvent in [CSM + 2x test cmpd] does not exceed 0.2% (*i.e.*, *minimally* a 500-fold dilution into CSM). This corresponds to a final assay concentration of 0.1% organic solvent.

NOTE 2.4 In *Step 6*, 100 μ l of the prepared [CSM + 2x test cmpd] will be added per well of the assay plate. If single-point assays are to be made, prepare a volume of each test compound media that is slightly greater than 100 μ l, thus allowing accurate volume transfers into respective assay wells. This assay kit provides 35 ml of CSM. Therefore, plan dilution schemes carefully so that the total volume of CSM required to perform dilutions of the positive- and negative-controls AND all test compounds does not exceed 35 ml.

ALTERNATIVE 2C: Antagonist Dose-Response Assays.

A common method of performing receptor inhibition studies is to prepare a co-mix of a known agonist (at a constant concentration typically between EC₅₀ – EC₈₅) AND a dilution series of 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 (Figure 1) that may be used to setup such receptor inhibition studies.

See *NOTE 2.0*.

Supplement a portion of **CSM-2** with an appropriate volume of aldosterone to generate a sufficient stock volume of [CSM + 2x aldosterone]. Use this [CSM + 2x aldosterone] stock to then prepare a 2x-concentrated dilution series of each test compound to be evaluated for antagonist activity. If desired, also prepare a dilution series of 2x-concentrated positive-control antagonist (supplied by the user).

NOTE 2.5 333 pM aldosterone typically corresponds to ~EC₇₅ in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used in setting up inhibition studies. Refer to *NOTE 2.2* for a suggested dilution scheme to prepare [CSM + 2x aldosterone].

NOTE 2.6 As a *general rule*: when an organic solvent is used to generate primary stock solutions of test compounds, we recommend that the user devise a dilution scheme so that the concentration of organic solvent in [CSM + 2x aldosterone + 2x test cmpd] does not exceed 0.2% (*i.e.*, *minimally* a 500-fold dilution into CSM). This corresponds to a final assay concentration of 0.1% organic solvent.

NOTE 2.7 When generating antagonist dose-response curves, it is recommended to perform all measurements in at least triplicate. In *Step 6*, 100 µl of [CSM + 2x aldosterone + 2x test cmpd] will be added per well of the assay plate. Therefore, devise an appropriate compound dilution scheme to yield a final volume of [CSM + 2x aldosterone + 2x test cmpd] that is slightly greater than 300 µl, thus allowing accurate volume transfers into each of the triplicate wells of the assay plate.

ALTERNATE 2D: HTS of Compounds for Antagonist Activities via Single-Point Assay

A common method of performing single-point receptor inhibition screens is to prepare a co-mix of a known agonist (at a concentration typically between EC₅₀ – EC₈₅) AND a single test concentration of the candidate antagonist compounds to be evaluated. This MR Reporter Assay System kit includes a 10 µM stock solution of **Aldosterone**, a potent agonist of MR (Figure 1) that may be used to setup such receptor inhibition screens.

See *NOTE 2.0*.

See *NOTE 2.5*.

Supplement a portion of **CSM-2** with an appropriate volume of aldosterone to generate an appropriate stock volume of [CSM + 2x aldosterone]. Refer to *NOTE 2.2* for a recommended dilution scheme to prepare [CSM + 2x aldosterone]. Use this stock of [CSM + 2x aldosterone] to then prepare a 2x-concentrated dilution of each test compound to be screened for antagonist activity. If desired, also prepare an appropriate dilution of 2x-concentrated positive-control antagonist (supplied by the user).

See *NOTE 2.6*.

NOTE 2.8 In *Step 6*, 100 µl of the prepared [CSM + 2x aldosterone + 2x test cmpd] will be added per well of the assay plate. If single-point assays are to be made, prepare a volume of each test compound media that is slightly greater than 100 µl, thus allowing accurate volume transfers into respective assay wells. This assay kit provides 35 ml of CSM. Therefore, plan dilution schemes carefully so that the total volume of [CSM + 2x aldosterone + 2x test cmpd] required to complete dilutions of the antagonist reference AND all test compounds does not exceed 35 ml.

- 3) Retrieve **MR Reporter Cells** from -80°C storage. Retrieve **CRM-1** from the 37°C water bath and sanitize the outside of the tube with a 70% ethanol swab. Transfer both tubes into a cell-culture hood. Perform a *rapid thaw* of the frozen cells by pipette-transferring 10.0 ml of the 37°C CRM-1 into the tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for at least 3 minutes.
- 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 Reporter Cells to disperse any cell aggregates. Transfer the entire volume of reporter cells into a sterile media basin. Use a multi-channel pipette to dispense 100 μl of cell suspension into each well of the **96-well Assay Plate**.

NOTE: Take special care to prevent cells from settling in the basin during the period of pipette-transfer. Lack of precision in transferring uniform volumes across the assay plate, and/or allowing cells to settle during the dispensing process, will cause well-to-well variation in the assay. For improved precision, speed, and ergonomic comfort, *the use of an electronic repeat-dispensing 8- or 12-channel pipette is recommended.*

- 6) Add 100 μl of 2x-concentrated treatment media (prepared in *Step 2*) to appropriate wells of the assay plate.
- 7) Replace the plate's lid and transfer it into a 37°C , humidified 5% CO_2 incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity environment within the cell culture incubator. This is necessary to prevent the onset of deleterious "edge-effects" in the assay plate.

- 8) For greater convenience on Day 2, retrieve **Detection Solutions I and II** from -80°C storage and place them in a dark refrigerator (4°C) to thaw overnight.

(Continue on DAY 2)

DAY 2: Subsequent manipulations do *not* require special regard for aseptic technique.

- 9) 30 minutes before intending to quantify MR activity, remove **Detection Solutions I** and **II** 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 Solutions I and II 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) Turn on the luminometer. Set the instrument to perform a single 5 second “plate shake” prior to reading the assay plate. Set the read time for 0.5 second per well.

NOTE: Many luminometers require a “warm up” period of 15 minutes *or more* before the photo-multiplier tube (PMT) attains maximal precision in reading very low luminescence signal.

- 11) *Immediately before proceeding to Step 12*, combine the contents of Detection Solutions I and II into a single tube to generate **Luciferase Detection Reagent (LDR)**. Mix gently to avoid foaming.

- 12) Following 22 - 24 hours of incubation, retrieve the assay plate from the incubator. Remove the plate’s lid and 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 liquid contents. Cells will remain tightly adhered to well bottoms.

- 13) Add 100 µl of room temperature **LDR** to each well of the assay plate.

NOTE: Pipette 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 may significantly degrade the accuracy and precision of the assay data. In the event of excessive bubble formation during processing, spin the assay plate (with lid) at *low speed* for 1-2 minutes using a room temperature centrifuge fitted with counter-balanced plate carriers.

- 14) Allow the assay plate to rest at room temperature for at least 15 minutes after the addition of LDR. Do not shake the assay plate during this period.

NOTE: See **APPENDIX 2** for information regarding signal stability.

- 15) Read the assay plate anytime between 15 - 90 minutes after adding LDR.

VI. Related Products

MR Family of 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
IB00501-B10	Human MR Reporter Assay System (Bulk Pack) Bulk Reagent Pack for 10x 96-well plates
IB00502	Human MR Reporter Assay System 1x 384-well format assays
IB00502-B10	Human MR Reporter Assay System (Bulk Pack) Bulk Reagent Pack for 10x 384-well plates
Alternative volumes of MR Assay Bulk Reagents can be custom manufactured. Please Inquire.	

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VII. Limited Use Disclosures

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans. Other applications of this product may require licenses from others, including one or more of the institutions listed below.

The method of recombinant expression of *Coleoptera* luciferase is covered by U.S. Patent 5,583,024. INDIGO Biosciences, Inc. has entered into a license agreement with The Regents of the University of California (Oakland, CA) for commercial application of the cDNA encoding the native luciferase of *Photinus pyralis*.

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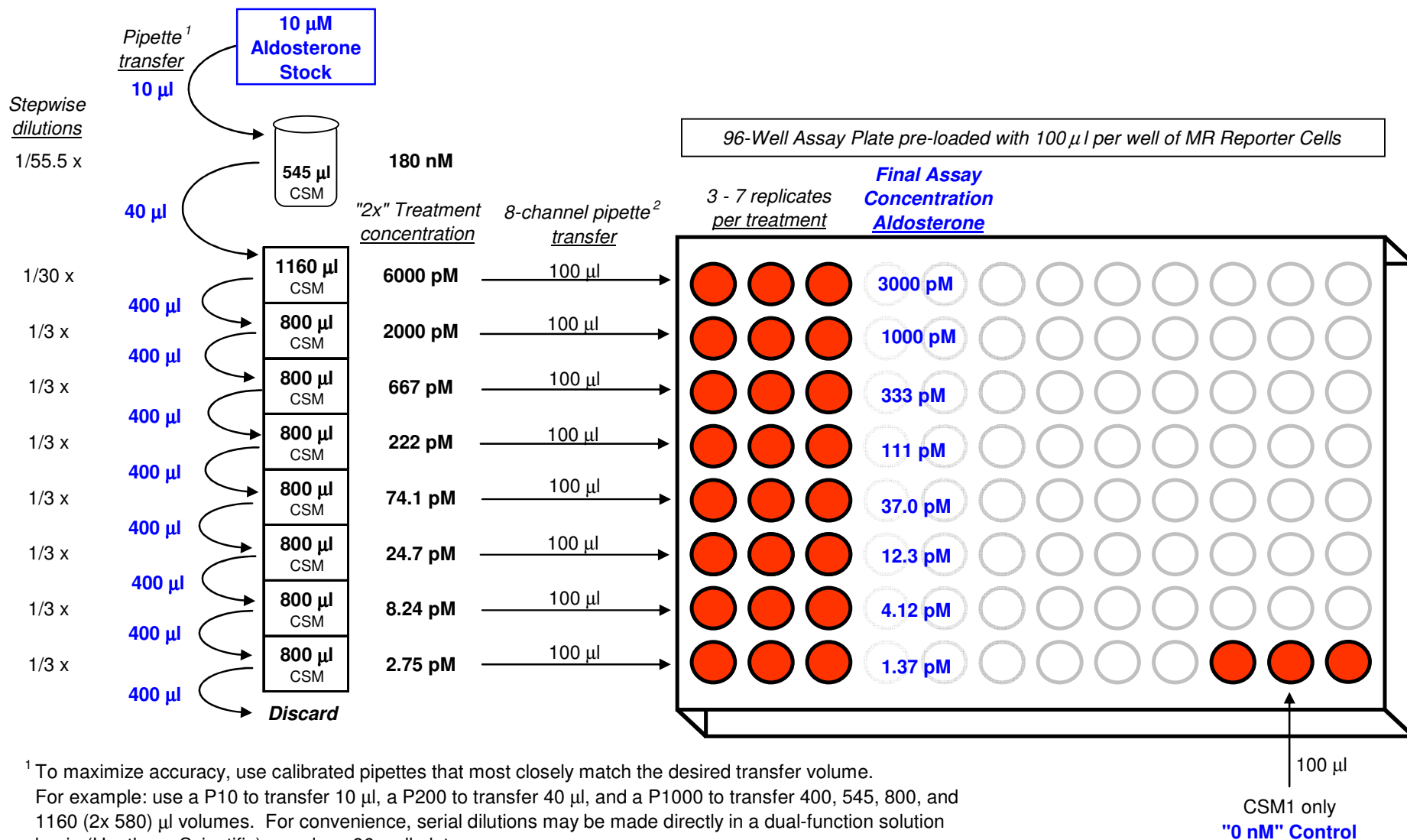
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APPENDIX 1

Example Dilution Scheme & Setup of MR Control Agonist Dose-Response Assay



¹ To maximize accuracy, use calibrated pipettes that most closely match the desired transfer volume. For example: use a P10 to transfer 10 µl, a P200 to transfer 40 µl, and a P1000 to transfer 400, 545, 800, and 1160 (2x 580) µl volumes. For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.

² To maximize speed, precision, and ergonomic comfort during manual pipetting of 100 µl volumes of reporter cells and treatment media, the use of an electronic, 8-channel, multi-dispensing P1000 pipette is recommended.

APPENDIX 2

Signal Stability of the Nuclear Receptor Reporter Assay

The human ER β Reporter Assay System is used here to demonstrate the light emission profile characteristic of INDIGO Biosciences' nuclear receptor assay system products.

As seen in **FIGURE 3**, between 5 and 15 minutes after adding LDR to assay wells the initial intensity of luminescence decays by 10-12%. However, luminescence signal stabilizes and remains essentially constant over the ensuing 75 minute reaction period. From T=15 minute to T=90 minutes, average luminescence measured from the same set of assay wells deviate by *less than 5%*

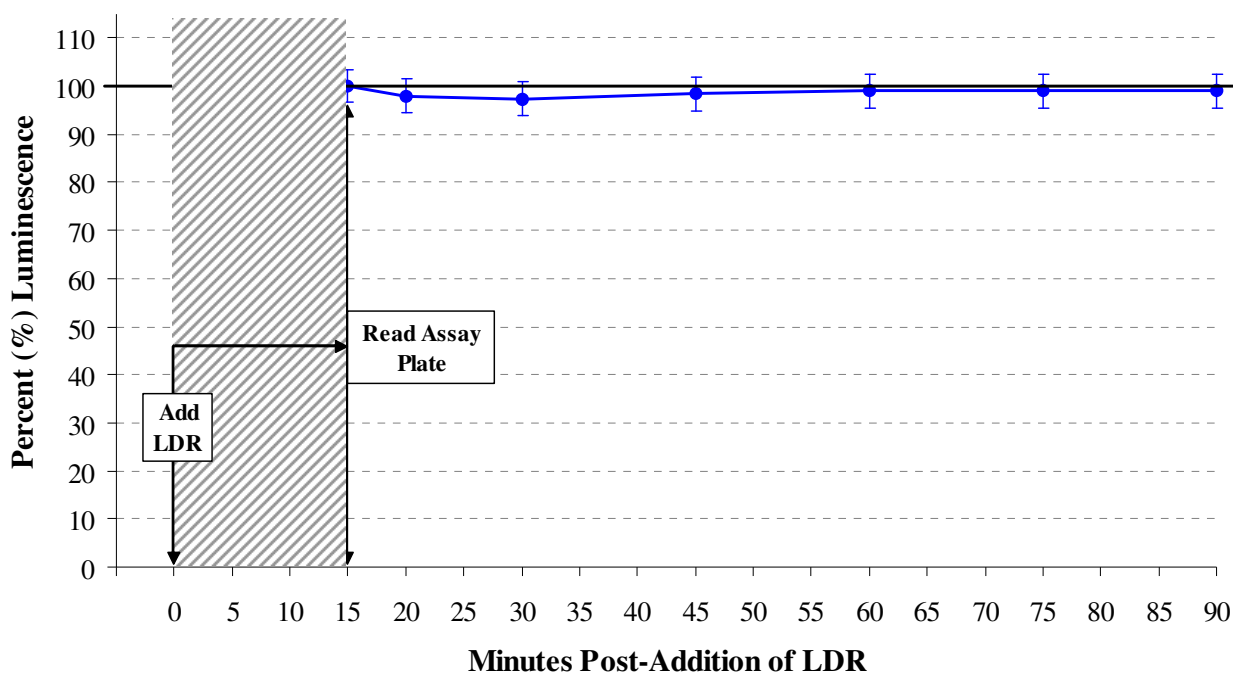


Figure 3. Stability of the luminescence signal. ER β reporter cells were cultured in 8 replicate wells of a 96-well assay plate, treated with 1 nM 17- β -estradiol for 24 hr, and media were replaced with LDR according to the protocol provided in Technical Manual #00411. Following an initial 5 minute rest period at room temperature, luminescence intensities were quantified by integrating light emission over 500 mSec. After the initial 5 minute time point, the assay plate was re-read at 10, 15, 20, 30, 45, 60, 75 and 90 minutes post-addition of LDR. Average RLU and respective standard deviation values were calculated, then normalized so that the luminescence signal at 15 minutes = 100%.

Allowing a *minimum* rest period of 15 minutes after the addition of LDR is particularly important for HTS users. Due to the logistics of batch-processing large numbers of assay plates, a significant time differential may occur between processing the first and last assay plates. Nonetheless, due to the stable emission profile of the luciferase reaction between 15 - 90 minutes, HTS users may be confident in comparing signal output from test samples in the first assay plate to those in the last plate in the stack.