



INDIGO Biosciences, Inc.

The Nuclear Receptor Company™

**Human Estrogen Receptor Alpha
(ER α ; ESR1; NR3A1)
Reporter Assay System**

96-well Format Assays
Product # IB00401

■

Technical Manual
(version 3.3)

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Human ER α Reporter Assay System 96-well Format Assays

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I. Description

▪ The Assay System ▪

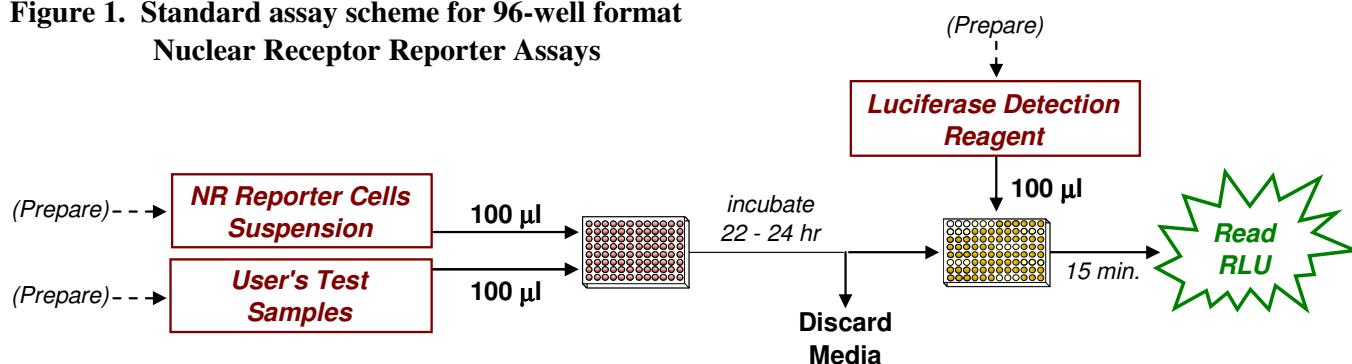
INDIGO Bioscience's Nuclear Receptor Reporter Assays are all-inclusive cell-based assay systems. In addition to nuclear receptor Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, a positive-control agonist, Luciferase Detection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

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 intermediate spin-and-wash steps, viability determinations, or cell titer adjustments.

This nuclear receptor assay system utilizes proprietary non-human mammalian cells engineered to provide constitutive, high-level expression of **Human Estrogen Receptor 1 (NR3A1)**, a ligand-dependent transcription factor commonly referred to as **ER α** . Because these cells incorporate a ER α -responsive luciferase reporter gene, quantifying expressed luciferase activity provides a sensitive surrogate measure of ER α activity in the treated cells. The primary application of this reporter assay system is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against human ER α .

As depicted in **Figure 1**, INDIGO's 96-well format nuclear receptor assay systems feature a streamlined assay protocol optimized to accommodate both low- and high-throughput plate-processing applications. 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 relative level of ER α activation in the reporter cells.

Figure 1. Standard assay scheme for 96-well format Nuclear Receptor Reporter Assays



▪ 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 O_2 and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i , CO_2 , and photon emission. Luminescence intensity of the luciferase reaction is quantified using a **luminometer**, and is reported in terms of Relative Light Units (RLU's).

INDIGO's 96-well format 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 for more information). Therefore, there is no requirement to sequentially process-and-read single 96-well format assay plates. However, when manually processing one or more assay plates, or when batch processing larger numbers of assay plates using an auto-dispenser, it is important to incorporate a 15 minute reaction-rest period to attain signal stability before quantifying RLU values. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared.

▪ Special NOTE: Considerations for Automated Dispensing ▪

In most cases, user's who run a small number of 96- or 384-well assay plates elect to process them manually using handheld electronic multi-channel pipettes. For applications involving large numbers of assay plates, the use of an automated dispensing instrument may be preferred, and beneficial.

When contemplating the use of an auto-dispenser first carefully considered the dead volume requirement of the instrument. "Dead volume" is defined as the minimum volume of reagent that is dedicated to the instrument for its setup and operation. In essence, this volume of reagent is consumed by the instrument; it will *not* be available for final dispensing into an assay plate.

Each of INDIGO's 96-well assay kits provide approximately **2.4 ml** of extra volume of **Reporter Cells suspension** (*i.e.*, Reporter Cells + CRM-1 combined) as well as **2.4 ml** of excess **Luciferase Detection Reagent** (*i.e.*, Detection Buffer + Detection Substrate combined).

The 2.4 ml volumes of excess reagents provided in a single 96-well assay kit will *not* be sufficient to meet the relatively large dead volume requirements of most "flow-through" type dispensers. Such instruments draw reagent from a reservoir and dispense through tubing, syringe and ejector head assemblies.

To satisfy the dead volume requirement of your intended auto-dispenser, pool respective reagents from the appropriate number of assay kits.

II. Product Components & Storage Conditions

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

The individual aliquots of ER α 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.

<u><i>Kit Components</i></u>	<u><i>Amount</i></u>	<u><i>Storage Temp.</i></u>
▪ ER α 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
▪ 17- β -estradiol, 10 μ M (in DMSO)	1 x 30 μ L	-20°C
(control agonist for ER α)	1 x 30 μ L	-20°C
▪ Detection Substrate	1 x 6.0 mL	-80°C
▪ Detection Buffer	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 ER α 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.

Human ER α Reporter Assay

17-beta-Estradiol Dose-Response, *Normalized*

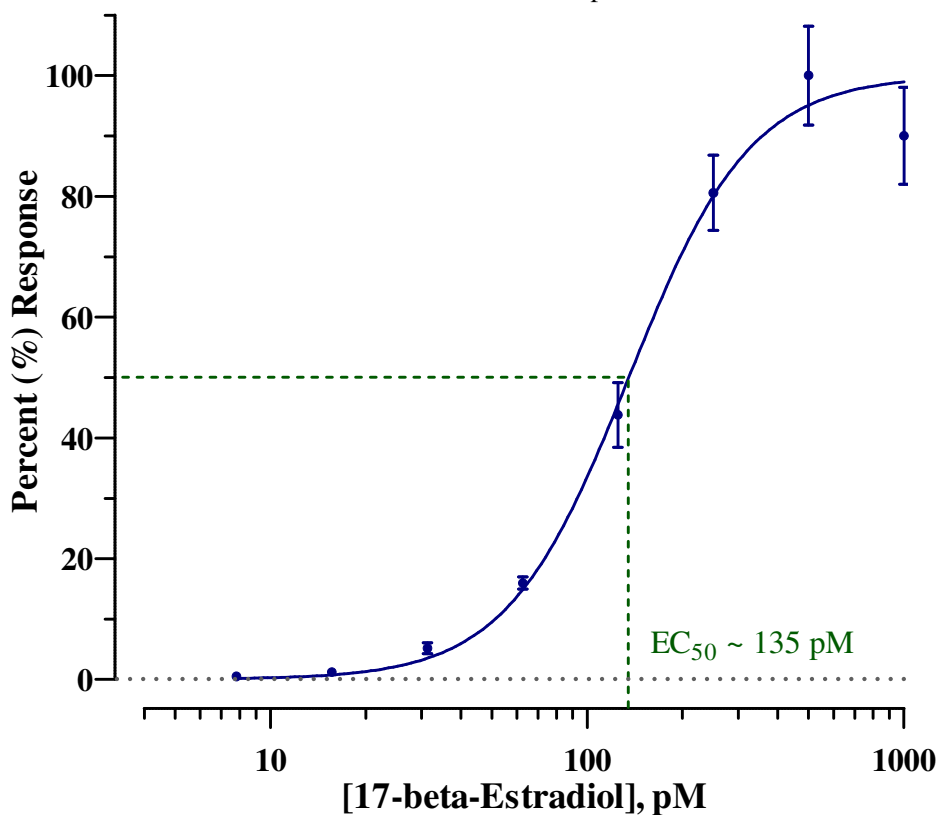


Figure 2. Agonist dose-response of the ER α Reporter Assay System.

ER α dose-response assays were performed according to the protocol provided in this Technical Manual. Reporter cells were treated with 17- β -estradiol at the following final concentrations: 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.81 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) 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. Error bars depict %CV. Dashed lines depict a representative EC₅₀ concentration of 17- β -estradiol in this ER α assay.

ER α Assay Performance at EC_{Max}

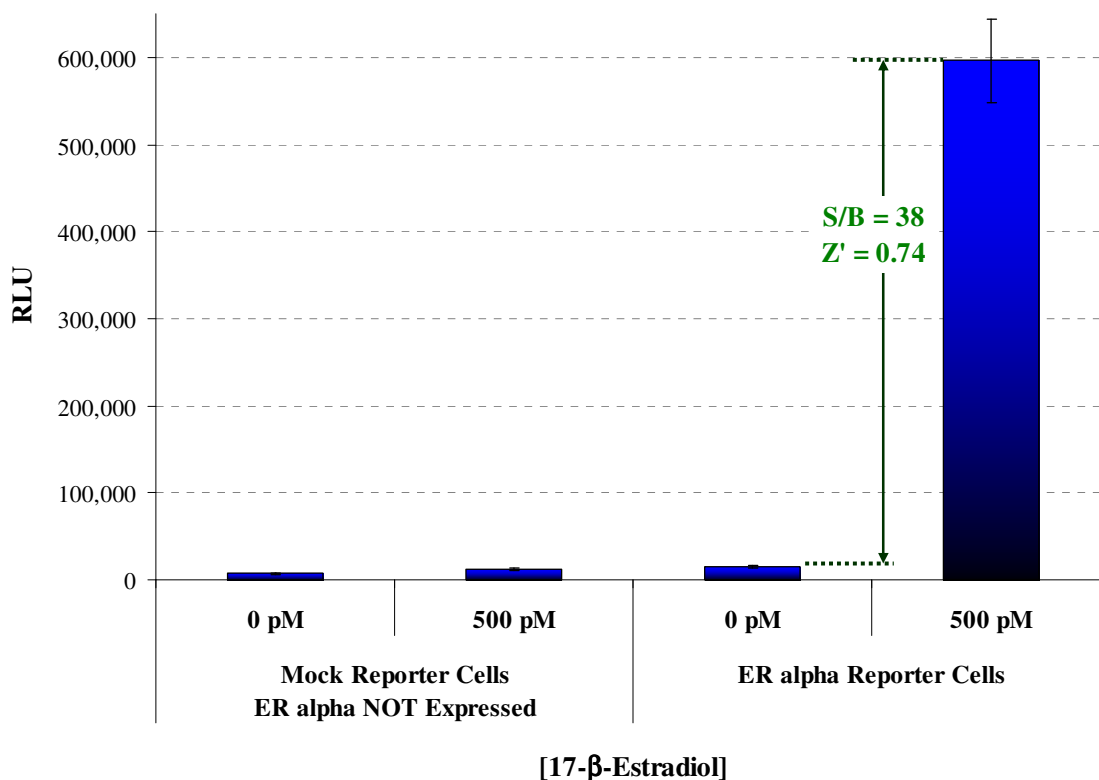


Figure 3. Validation of the ER α reporter assay for screening applications.

ER α reporter cells were treated with media alone, or media supplemented with 500 pM 17- β -estradiol. 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 ER α expression vectors. Mock reporter cells are not provided with assay kits). Mock reporter cells and the ER α reporter cells were cultured, treated with 17- β -estradiol, and processed in identical manner. A minimum of six replicate assay wells were processed for each treatment condition. 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: ER α reporter cells treated with 500 pM 17- β -estradiol yielded S/B = 38 and a calculated Z' value of 0.74. Similarly treated mock reporter cells demonstrate only low levels of background luciferase activity (< 2% that of the reporter cells at EC_{Max}). Thus, luminescence results from ligand-activation of the exogenous human ER α expressed in these reporter cells. These data confirm the robust performance of this ER α 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 ≤ 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 2)

Use **CSM-2** to prepare a dilution series of 2x-concentrated control agonist (17- β -estradiol) AND an appropriate dilution series of 2x-concentrated test compound(s) to be assayed.

NOTE 2.0 In *Step 6*, 100 μ l of the prepared [CSM + test cmpd] is added into an assay well already containing 100 μ l 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 μ l 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 μ l, thus allowing accurate volume transfers into each of the triplicate wells of the assay plate.

NOTE 2.2 When using 17- β -estradiol as the positive-control agonist, we find the following assay concentration range provides a complete dose-response: 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.81, and 0 picoMolar (pM; 10^{-12} Molar), as depicted in **Figure 2**. 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 17- β -estradiol may be achieved by following the example presented in **APPENDIX 1**. In brief, use a portion of CSM to first perform a 100-fold dilution of the provided 10 μ M 17- β -estradiol stock (*e.g.*, mix 8.0 μ l 17- β -estradiol stock with 792 μ l of CSM). Use this intermediate stock to perform a subsequent 50-fold dilution to achieve the first desired 2x-concentration of 2000 pM 17- β -estradiol (containing 0.02% DMSO). Continue by using a portion of the 2000 pM solution to perform seven sequential 2-fold dilutions to produce 1000, 500, 250, 125, 62.5, 31.2, and 15.6 pM 2x-concentrated stocks. Neat CSM (or CSM supplemented with 0.02% DMSO; *i.e.*, the highest concentration of “vehicle”) may be used to provide the “0 pM” 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 ER α Reporter Assay System kit includes a 10 μ M stock solution of 17- β -estradiol, a potent agonist of ER α that may be used as a positive-control. An assay concentration of 500 pM 17- β -estradiol typically provides $\geq 95\%$ activation of ER α (**Figure 2**). Hence, 1000 pM 17- β -estradiol 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 17- β -estradiol 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 ER α Reporter Assay System kit includes a 10 μ M stock solution of 17- β -estradiol, a potent agonist of ER α (**Figure 2**) 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 17- β -estradiol to generate a sufficient stock volume of [CSM + 2x 17- β -estradiol]. Use this [CSM + 2x 17- β -estradiol] 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 2.5 150 pM 17- β -estradiol typically corresponds to ~EC₇₀ 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 17- β -estradiol].

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 17- β -estradiol + 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 μ l of [CSM + 2x 17- β -estradiol + 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 17- β -estradiol + 2x test cmpd] that is slightly greater than 300 μ l, 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 compound(s) to be evaluated. This ER α Reporter Assay System kit includes a 10 μ M stock solution of 17- β -estradiol, a potent agonist of ER α (**Figure 2**) 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 17- β -estradiol to generate an appropriate stock volume of [CSM + 2x 17- β -estradiol]. Refer to *NOTE 2.2* for a recommended dilution scheme to prepare [CSM + 2x 17- β -estradiol]. Use this stock of [CSM + 2x 17- β -estradiol] 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 μ l of the prepared [CSM + 2x 17- β -estradiol + 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 + 2x 17- β -estradiol + 2x test cmpd] required to complete dilutions of the antagonist reference AND all test compounds does not exceed 35 ml.

- 3) Retrieve **ER α 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) Invert the tube of Reporter Cells several times to disperse any cell aggregates and to gain an homogenous cell suspension. Transfer the entire volume of reporter cells into a sterile media basin. Use a multi-channel pipette to dispense 100 μ l 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 μ l 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% CO₂ incubator for 22 - 24 hours.
- NOTE: Ensure a high-humidity ($\geq 90\%$) 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 Substrate and Detection Buffer** 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 ER α 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 the 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) 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 entire volumes of Detection Substrate and Detection Buffer 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 μ l 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

ERα Family of Assay Products	
<i>Product No.</i>	<i>Product Descriptions</i>
IB00401-32	Human ER α Reporter Assay System 3x 32 assays in 96-well format
IB00401-48	Human ER α Reporter Assay System 2x 48 assays in 96-well format
IB00401	Human ER α Reporter Assay System 1x 96-well format assay
IB00401-B10	Human ER α Reporter Assay System (Bulk Pack) Bulk Reagent Pack for 10x 96-well plates
IB00402	Human ER α Reporter Assay System 1x 384-well format assays
IB00402-B10	Human ER α Reporter Assay System (Bulk Pack) Bulk Reagent Pack for 10x 384-well plates
Alternative volumes of Assay Bulk Reagents may be custom manufactured to better accommodate Customer needs. Please Inquire.	

Panel of ER Assays	
<i>Product No.</i>	<i>Product Description</i>
IB00421-48P	Human ER α and ER β Reporter Assay PANEL 48 assays each in 1x 96-well plate

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 assay plate formats
LCM-05	Reagent in 5x-bulk volume to perform 480 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats
LCM-10	Reagent in 10x-bulk volume to perform 960 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats

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*.

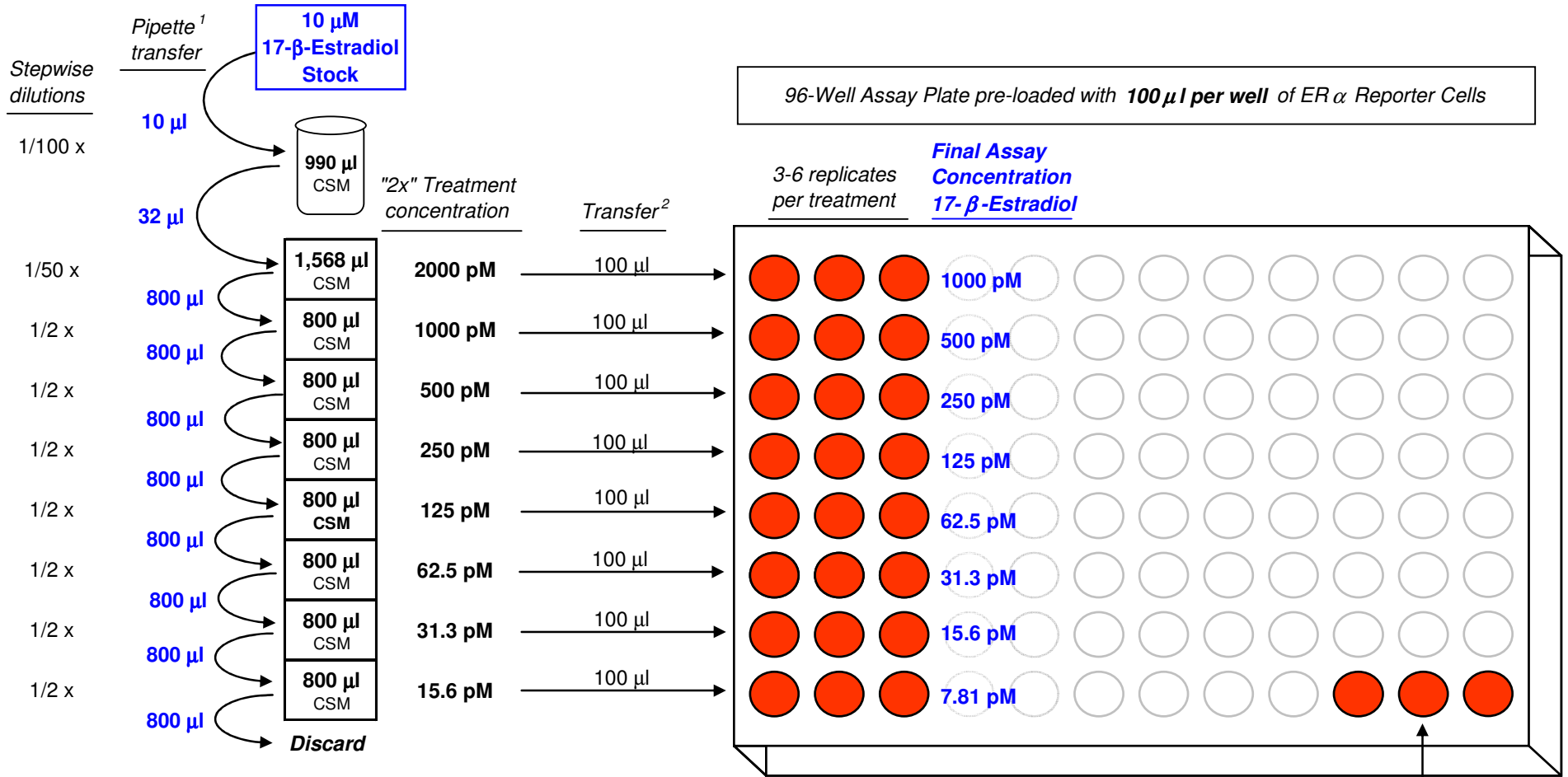
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APPENDIX 1

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



¹ 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 4**, 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%*

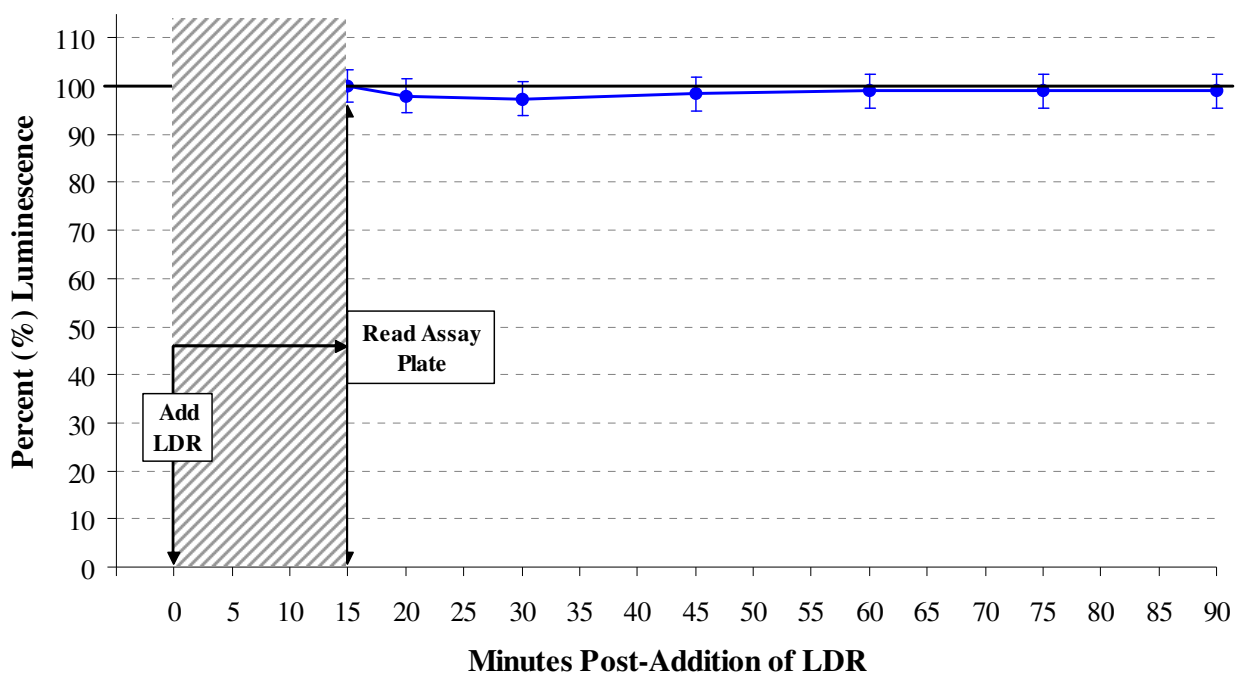


Figure 4. Stability of the luminescence signal. ER β reporter cells were cultured in 8 replicate wells of a 96-well assay plate, treated with 1 nM 17- β -estradiol for 24 hr, and media were replaced with LDR according to the protocol provided in Technical Manual #00411. Following an initial 5 minute 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.