



**INDIGO Biosciences, Inc.**

The Nuclear Receptor Company™

**Human Estrogen Receptor Alpha  
(ER $\alpha$ ; ESR1; NR3A1)  
Reporter Assay System**

**384-well Format Assays**  
Product # IB00402

■

**Technical Manual**  
*(version 4.0)*

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## Human ER $\alpha$ Reporter Assay System 384-well Format Assays

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## I. Description

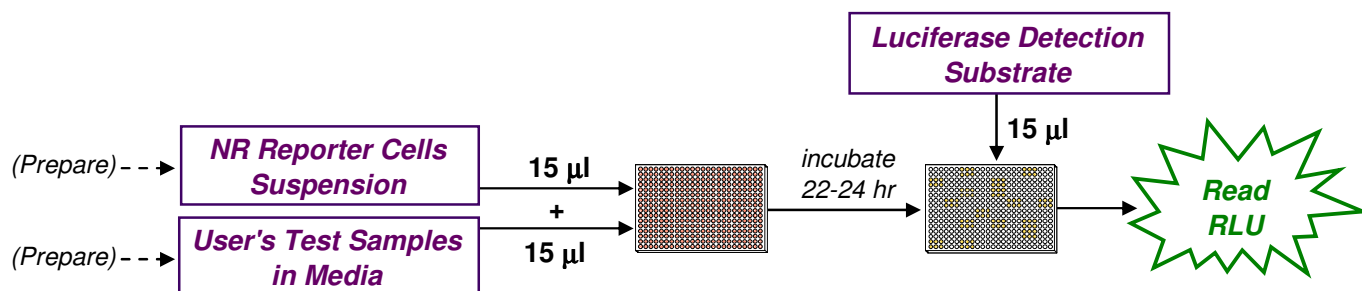
### The Assay System

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 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 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 alpha, or simply **ER $\alpha$** . Because these cells incorporate an ER $\alpha$ -responsive luciferase reporter gene, quantifying expressed luciferase activity provides a sensitive surrogate measure of ER $\alpha$  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 $\alpha$ .

As depicted in Figure 1, INDIGO's 384-well format assays feature a streamlined, 'homogenous' assay procedure. The protocol minimizes plate handling steps to best accommodate fully automated high-throughput plate-processing. In brief: On Day 1, Reporter Cells and the user's test samples are sequentially dispensed into the assay plate, which is then incubated overnight. On Day 2, Detection Substrate is added into assay wells and the intensity of luminescence signal from each luciferase reaction is quantified.



**Figure 1. Homogenous Assay Chemistry optimal for HTS.**

Unlike INDIGO's 96-well format assays, this HTS protocol for processing 384-well assay plates does *not* require a 'media discard' step.

## ▪ 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 reaction is quantified using a plate-reading **luminometer**, and is reported in terms of Relative Light Units (RLU).

All of INDIGO's 384-well format Nuclear Receptor Reporter Assay Systems feature a luciferase detection reagent specially formulated to provide stable light emission between 30 and 105<sup>+</sup> minutes after initiating the luciferase reaction (see APPENDIX 2). Therefore, users who choose to manually process only one or two assay plates, or to batch process numerous assay plates at one time, will need to incorporate a 30 minute reaction-rest period prior to measuring RLU values. Thus, luminescence signal from all sample wells within a given plate, as well as sample wells from one plate to the next, may be directly compared.

### ▪ Special NOTE: Considerations for Automated Dispensing ▪

When processing a small number of assay plates, first carefully considered the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. "Dead volume" is defined as the minimum volume of reagent that is dedicated to the instrument for 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.

INDIGO's 384-well assay kits provide approximately **2 ml** of extra volume of **Reporter Cells suspension** (*i.e.*, Reporter Cells + CRM-1 combined) as well as **2 ml** of excess **Detection Substrate**. Hence, the reagent volumes provided in a single 384-well assay kits are sufficient for researchers wishing to:

- 1.) dispense using *low dead volume* automated dispensing instruments. Such instruments are variously referred to as "aspiration-" or "acoustic droplet ejection-" type dispensing instruments; or
- 2.) perform *manual* dispensing from a conventional media basin. With moderate practice and skill in the use of an 8- or 12-channel electronic repeat-dispensing pipette, manual dispensing into a single 384-well plate can be accomplished quickly and with high precision.

In most cases, the ~ 2 ml volumes of excess reagents provided in a single assay kit will *not* be sufficient to meet the relatively large dead volume requirements of "flow-through" type dispensers. Such instruments draw reagent from a reservoir and dispense through tubing, syringe and ejector head assemblies.

## ***II. Product Components & Storage Conditions***

This Human ER $\alpha$  Reporter Assay System contains materials to perform assays in a single 384-well plate.

The individual aliquots of ER $\alpha$  Reporter Cells, CRM-1 and Detection Substrate are provided as single-use consumable reagents. Each reagent is supplied with sufficient extra volume to accommodate low-dead volume auto-dispensers or manual dispensing (see *Special Note* on pg 4). However, these excess reagents should be discarded after assay set-up. Once thawed, Reporter Cells can NOT be refrozen, nor can they be maintained in prolonged culture with any hope of retaining downstream assay performance.

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. In particular, *Reporter Cells must be maintained at a continuous -80°C storage temperature* until immediately prior to use.

The date of product expiration is printed on the Product Qualification Insert (PQI) enclosed with each kit.

<b><i><u>Kit Components</u></i></b>	<b><i><u>Amount</u></i></b>	<b><i><u>Storage Temp.</u></i></b>
▪ ER $\alpha$ Reporter Cells	1 x 4.0 mL	<b>-80°C</b>
▪ Cell Recovery Medium 1 (CRM-1)	1 x 3.8 mL	-20°C
▪ Compound Screening Medium 2 (CSM-2)	1 x 35 mL	-20°C
▪ 17- $\beta$ -estradiol, 10 $\mu$ M (in DMSO) (control agonist for ER $\alpha$ & ER $\beta$ )	1 x 30 $\mu$ L	-20°C
▪ Detection Substrate	1 x 7.8 mL	<b>-80°C</b>
▪ 384-well assay plate (white, sterile, cell-culture ready)	1	ambient

### ***III. Alternative Applications for this Reporter Assay***

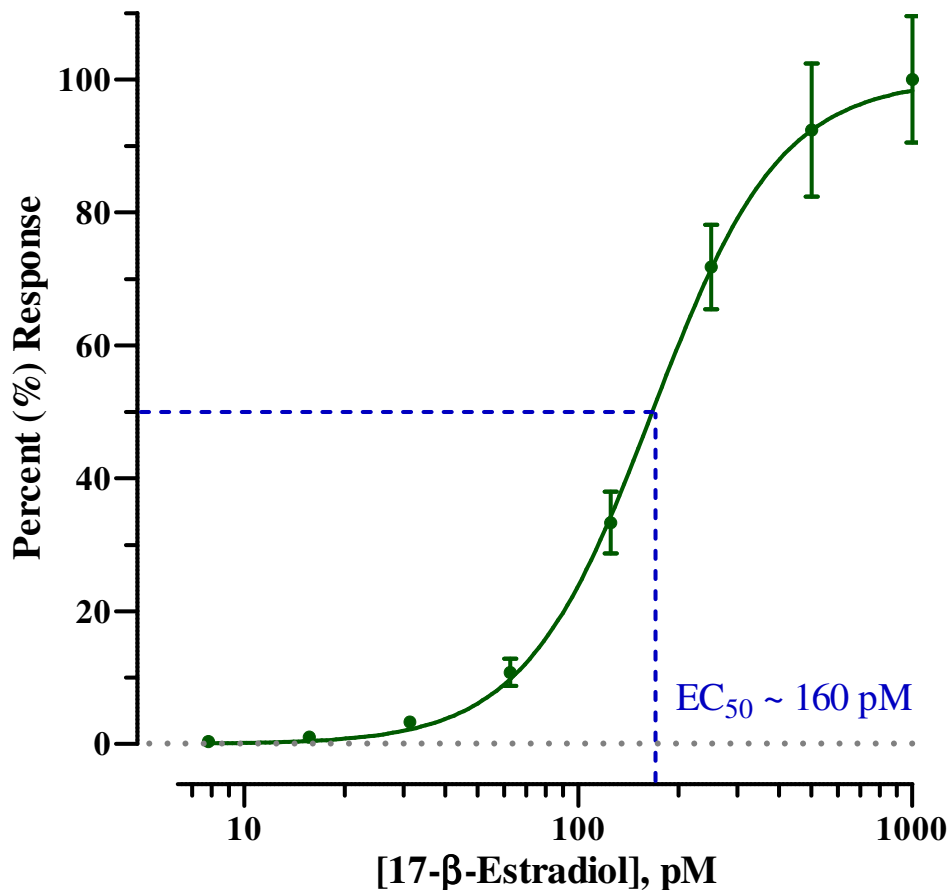
This nuclear receptor reporter assay system is a sensitive and versatile research tool. As such, the user may configure these assays in several ways to achieve different research objectives.

The “Assay Procedure” begins in Section V. It provides specific instructions for performing each ER $\alpha$  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-response analyses (Alternate 2A) or antagonist dose-response analyses (Alternate 2C), or to perform single-point screening of test compounds for either agonist activities (Alternate 2B) or antagonist activities (Alternate 2D).

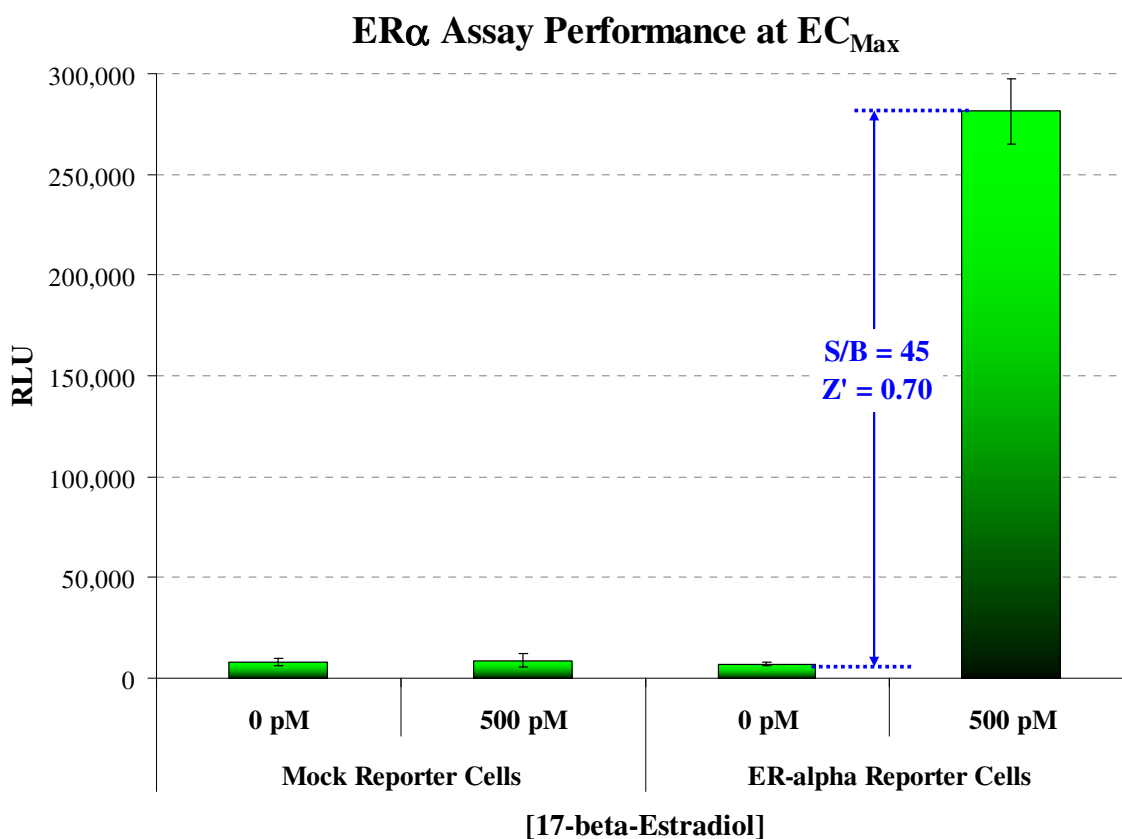
Due to the experiment-specific nature of these steps, these alternative procedures (and their attendant “NOTES”) are intended to serve as guidelines only. They are offered to assist researchers in formulating an assay design that is best suited to achieve their specific research goals.

# Human ER $\alpha$ Reporter Assay

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



**Figure 2. Agonist dose-response of the ER $\alpha$  Reporter Assay System.** ER $\alpha$  dose-response assays were performed according to the protocol provided in this Technical Manual. Reporter cells were treated with 17- $\beta$ -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 Detection Substrate was applied 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. Error bars depict %CV. Dashed lines depict a representative EC<sub>50</sub> concentration of 17- $\beta$ -estradiol in this ER $\alpha$  assay.



**Figure 3. Validation of the ER $\alpha$  reporter assay for screening applications.**

ER $\alpha$  reporter cells were treated with media alone, or media supplemented to yield a final assay concentration of 500 pM 17- $\beta$ -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 $\alpha$  expression vector. Mock reporter cells are not provided with assay kits). Mock reporter cells and the ER $\alpha$  reporter cells were cultured, treated with 17- $\beta$ -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)<sup>1</sup>.

*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:** ER $\alpha$  reporter cells treated with 500 pM 17- $\beta$ -estradiol yielded S/B = 45 and a calculated Z' value of 0.70. Similarly treated mock reporter cells demonstrate only low levels of background luciferase activity (< 2% that of the reporter cells at EC<sub>Max</sub>). Thus, luminescence results from ligand-activation of the exogenous human ER $\alpha$  expressed in these reporter cells. These data confirm the robust performance of this ER $\alpha$  Reporter Assay System, and demonstrate its suitability for use in HTS applications.<sup>1</sup>

<sup>1</sup> 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% CO<sub>2</sub> 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*).
- For *manual* dispensing:
  - disposable media basin, sterile (*Step 5*).
  - 8- *or* 12-channel pipette & sterile tips appropriate for the transfer of 15 µl 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 9-11* 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 temperature equilibration of CSM-2.
- Warm **CRM-1** to 37°C using a water bath. Pre-warmed CRM-1 is required in *Step 3*.

*NOTE 1:* For greater convenience, the user may transfer the tubes of frozen CRM-1 and CSM-2 into a refrigerator on the day *preceding* assay setup, thus allowing the reagents to thaw overnight.

**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 AND an appropriate dilution series of 2x-concentrated test compound(s) to be assayed.

*NOTE 2.0* In *Step 6*, 15 µl of the prepared [CSM + test cmpd] is added into an assay well already containing 15 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*, 15 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 45 µl, 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<sup>-12</sup> 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 µM 17-β-estradiol stock (e.g., mix 8.0 µl 17-β-estradiol stock with 792 µl 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 $\alpha$  Reporter Assay System kit includes a 10  $\mu$ M stock solution of 17- $\beta$ -estradiol, a potent agonist of ER $\alpha$  that may be used as a positive-control. An assay concentration of 500 pM 17- $\beta$ -estradiol typically provides  $\geq 95\%$  activation of ER $\alpha$  (Figure 2). Hence, 1000 pM 17- $\beta$ -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- $\beta$ -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 compd] 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*, 15  $\mu$ l of the prepared [CSM + 2x test compd] 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 15  $\mu$ 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 EC<sub>50</sub> – EC<sub>85</sub>) AND a dilution series of the test compound(s) to be evaluated for antagonist activity. This ER $\alpha$  Reporter Assay System kit includes a 10  $\mu$ M stock solution of 17- $\beta$ -estradiol, a potent agonist of ER $\alpha$  (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- $\beta$ -estradiol to generate a sufficient stock volume of [CSM + 2x 17- $\beta$ -estradiol]. Use this [CSM + 2x 17- $\beta$ -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- $\beta$ -estradiol typically corresponds to ~EC<sub>70</sub> 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- $\beta$ -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- $\beta$ -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*, 15  $\mu$ l of [CSM + 2x 17- $\beta$ -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- $\beta$ -estradiol + 2x test cmpd] that is slightly greater than 45  $\mu$ 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 EC<sub>50</sub> – EC<sub>85</sub>) AND a single test concentration of the candidate antagonist compound(s) to be evaluated. This ER $\alpha$  Reporter Assay System kit includes a 10  $\mu$ M stock solution of 17- $\beta$ -estradiol, a potent agonist of ER $\alpha$  (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- $\beta$ -estradiol to generate an appropriate stock volume of [CSM + 2x 17- $\beta$ -estradiol]. Refer to *NOTE 2.2* for a recommended dilution scheme to prepare [CSM + 2x 17- $\beta$ -estradiol]. Use this stock of [CSM + 2x 17- $\beta$ -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*, 15  $\mu$ l of the prepared [CSM + 2x 17- $\beta$ -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 15  $\mu$ 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- $\beta$ -estradiol + 2x test cmpd] required to complete dilutions of the antagonist reference AND all test compounds does not exceed 35 ml.

- 3) Retrieve **ER $\alpha$  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 the entire 3.8 ml volume 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. The resulting volume of cell suspension is 7.8 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) Invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Transfer the entire volume of reporter cells suspension into a sterile media basin. Use a multi-channel pipette to dispense 15  $\mu$ l of cell suspension into each well of the **384-well Assay Plate**.

*NOTE 5:* 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. If the user elects to manually dispense reagents, *the use of an electronic repeat-dispensing 8- or 12-channel pipette is recommended.*

- 6) Add 15  $\mu$ 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% CO<sub>2</sub> incubator for 22 - 24 hours.
- NOTE 7:* Ensure a high-humidity ( $\geq 90\%$ ) 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 -80°C storage and place it 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) Retrieve **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 9:* 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 and temperature equilibration.

- 10) Turn on the luminometer. Set the instrument to perform a single 5 second “plate shake” prior to reading the assay plate, and a read time of 0.5 second per well.

*NOTE 10:* Many luminometers require a “warm up” period of at least 15 minutes before the photo-multiplier tube (PMT) attains maximal precision in reading very low luminescence signal. Some instruments are capable of performing accurate and precise measurements using read times of less than 0.5 second per well.

- 11) The user’s intended method of further processing the Assay Plate(s) will determine which of the two Alternative Step 11 protocols (page 17) will be used.

*NOTE 11:* As discussed in APPENDIX 2, the luminescent signal is unstable during the first 30 minutes of the luciferase reaction, and will experience  $\geq 35\%$  loss in intensity. However, after the initial 30 minute reaction period the luminescence signal achieves a stable emission profile.

*Alternative Step 11A* should be used when plates are to be processed in any way that results in a significant time differential ( $\geq 60$  seconds) between adding Detection Substrate into the *first* and *last* wells of the assay plate(s), OR there is a significant time differential ( $\geq 60$  seconds) between reading the first and last wells on one or more assay plates. Examples include manual processing of assay plates, *or* the use of an automated instrument to batch-process multiple assay plates, *or* the use of a luminometer limited to reading only one assay well at a time, thereby requiring an elapsed time of  $\geq 60$  seconds to read the first and last wells on the assay plate. Under all such handling conditions, it is necessary to incorporate a 30 minute reaction-rest period after Detection Substrate is dispensed. This allows the luminescent signals from all wells, on all assay plates, to attain a stable light emission profile prior to quantifying RLU values.

*Alternative Step 11B* should be followed only when using fully automated, high-throughput plate handling. Further, *a.*) multiple assay plates are processed individually in a time-consistent “plate dispense-and-read” manner, AND *b.*) there is less than a 1 minute time differential between dispensing Detection Substrate across the assay plate, AND *c.*) there is less than a 1 minute time differential between reading the first and last wells on the assay plate. Under such circumstances, the well-to-well differential in luminescence due to signal decay will be insignificant, and incorporating a reaction-rest period prior to quantifying RLU values is not necessary.

**Alternative 11A.)** Used if assay plates are manually processed, *or* multiple assay plates are batch-processed, *or* a plate-reading luminometer is used that requires more than 60 seconds to read all wells of the 384-well assay plate (refer to *NOTE 11*).

- i.) Following 22 - 24 hours of incubation, retrieve the assay plate from the incubator. Remove the plate's lid. Add 15  $\mu$ l of **Detection Substrate** to each well of the assay plate.

*NOTE: Perform manual reagent transfers 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 manual 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.

- ii.) Allow the plate(s) to rest at room temperature for **30 minutes**. Do not shake the assay plate(s) during this period.
- iii.) Place the assay plate in the luminometer and quantify luminescence.

~ *or* ~

**Alternative 11B.)** Used if numerous assay plates are to be individually processed using a fully automated high-speed plate handling system. It is imperative to use a plate-to-plate process that ensures rapid reagent dispensing, and followed (at a consistent time interval) by rapid plate-reading (refer to *NOTE 11*).

- i.) Following 22 - 24 hours of incubation, retrieve all assay plates from the incubator. Remove plate lids.
- ii.) Dispense 15  $\mu$ l of **Detection Substrate** into each well of assay plate #1.
- iii.) Transfer the assay plate #1 to a luminometer and quantify luminescence.
- iv.) Repeat the "dispense-and-read" process for assay plate #2, and for each individual assay plate thereafter.

## *VI. Related Products*

<b>ER<math>\alpha</math> Family of Assay Products</b>	
<i><b>Product No.</b></i>	<i><b>Product Descriptions</b></i>
IB00401-32	Human ER $\alpha$ Reporter Assay System 3x 32 assays in 96-well format
IB00401-48	Human ER $\alpha$ Reporter Assay System 2x 48 assays in 96-well format
IB00401	Human ER $\alpha$ Reporter Assay System 1x 96-well format assay
IB00401-B10	Human ER $\alpha$ Reporter Assay System (Bulk Pack) Bulk Reagent Pack for 10x 96-well plates
IB00402	Human ER $\alpha$ Reporter Assay System 1x 384-well format assays
IB00402-B10	Human ER $\alpha$ 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.	

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

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

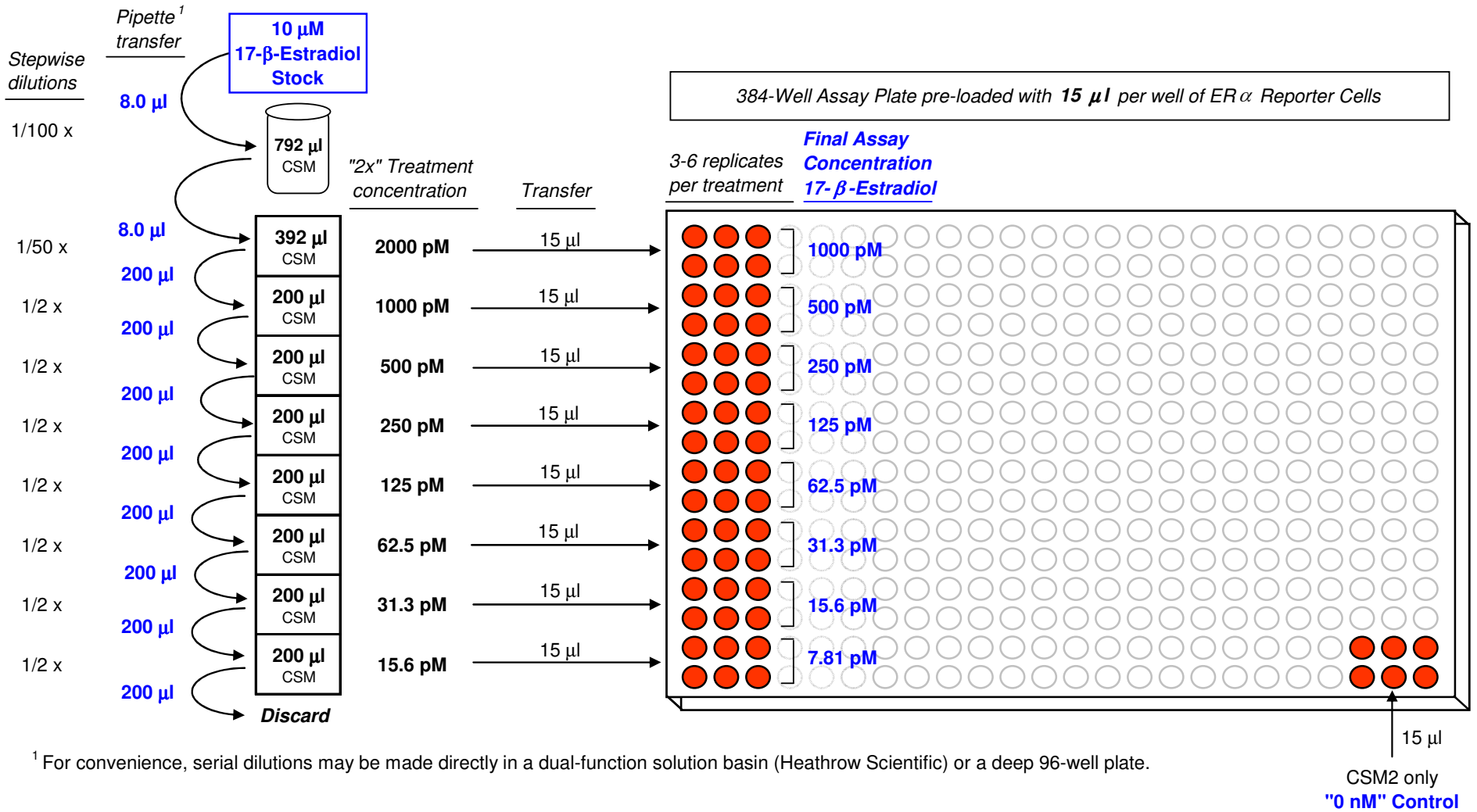
“CryoMite” is a Trademark <sup>™</sup> of INDIGO Biosciences, Inc.

Product prices, availability, specifications and claims are subject to change without prior notice.

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## APPENDIX 1

### Example Dilution Scheme & Setup of ER $\alpha$ Control Agonist Dose-Response Assay



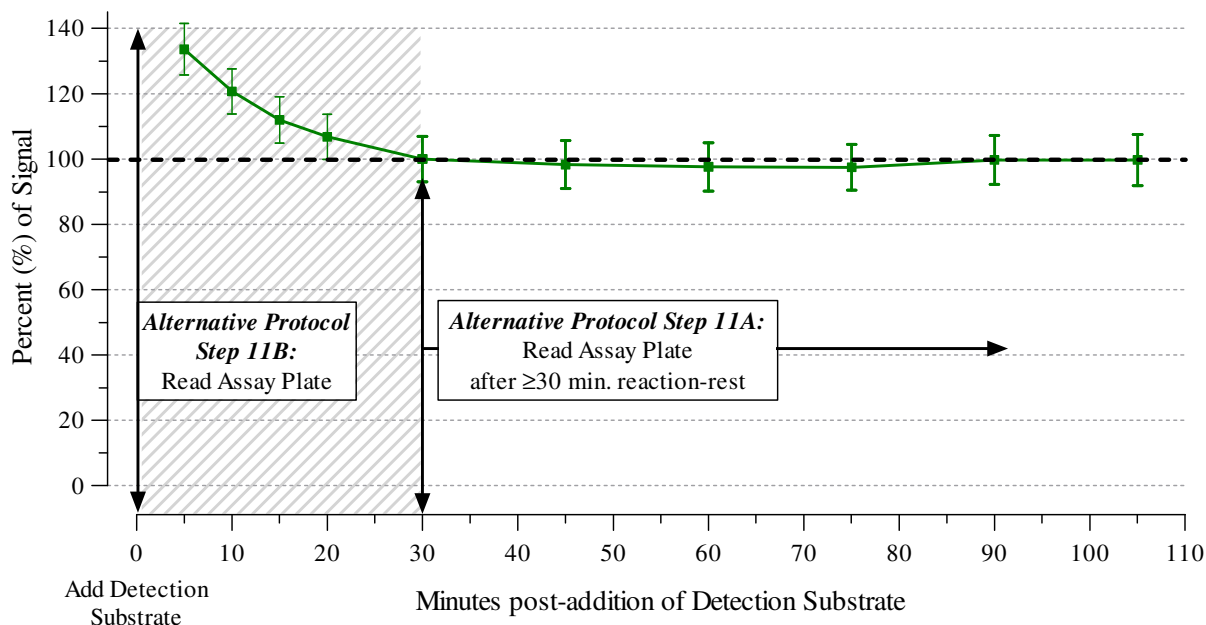
## APPENDIX 2

### Signal Stability of the Nuclear Receptor Reporter Assay

The Human PPAR $\alpha$  Reporter Assay System is used here to demonstrate the light emission profile of INDIGO Biosciences' homogenous assay in 384-well format.

As seen in **FIGURE 4**, within the first 30 minutes after dispensing Detection Substrate into 384-well assay plates the intensity of the luminescent reaction decays, at a variable rate, by  $\geq 35\%$ . After 30 minutes, however, the reaction stabilizes and signal intensity remains constant for, minimally, the ensuing 75 minute reaction period. From T=30 minutes to T=105 minutes, average RLU values measured from the same assay wells deviate by *less* than 3%.

Allowing a reaction-rest period of 30 minutes after the addition of Detection Substrate is important for users who elect to manually process a 384-well assay plate, *or* batch-process multiple assay plates using an automated dispenser, *or* use a luminometer that requires more than 60 seconds to read the entire assay plate. In each of these situations a significant time differential will occur between processing and/or reading the first assay well on the first plate and the last assay well on the last plate. Nonetheless, by incorporating a 30 minute reaction-rest period prior to measuring RLU values, users may be confident in directly comparing signal data from all sample wells, from the first assay plate to the last plate in the stack.



**Figure 4. Stability of the luminescence signal from 384-well format, homogenous assay protocol.** PPAR $\alpha$  reporter cells were cultured in a 384-well assay plate (n=8) in the presence of 100 nM GW590735. After 24 hr incubation, Detection Substrate was added into assay wells and the assay plate was allowed to rest at room temperature. At 5, 10, 15, 20, 30, 45, 60, 75, 90 and 105 minutes post-addition of Detection Substrate, luminescence intensities were quantified by integrating light emission over 500 mSec. Average RLU values were calculated, then normalized so that the luminescence signal at 30 minutes = 100%.