Human Estrogen Receptor Alpha
(ERα; ESR1; NR3A1)
Reporter Assay System

3x 32 Assays in 96-well Format
Product # IB00401-32

Technical Manual
(version 6.0)

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Human ERα Reporter Assay System
3x 32 Assays in 96-well Format

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I. Description

• The Assay System •

This nuclear receptor assay system utilizes proprietary non-human cells engineered to provide constitutive, high-level expression of the full-length Human Estrogen Receptor 1 (NR3A1), a ligand-dependent transcription factor commonly referred to as ERα.

INDIGO’s Reporter Cells include the luciferase reporter gene functionally linked to an ERα-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in ERα activity. Luciferase gene expression occurs after ligand-bound ERα 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 target 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.

ERα Reporter Cells are prepared using INDIGO’s proprietary CryoMite™ process. This cryo-preservation method yields exceptional 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, cell titer adjustments, or the pre-incubation of reporter cells prior to assay setup.

The principle application of this assay product is in the screening of test samples to quantify functional activities, either agonist or antagonist, that they may exert against the human estrogen receptor. It is an all-inclusive assay system that includes, in addition to ERα Reporter Cells, two optimized media for use during cell culture and in diluting the test samples, a reference agonist, Luciferase Detection Reagent, a cell culture-ready assay plate, and a detailed protocol.

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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 Mg²⁺-dependent reaction that consumes O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PPi, CO₂, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer, and is reported in terms of Relative Light Units (RLU’s).

INDIGO’s Nuclear Receptor Reporter Assay Systems feature a luciferase detection reagent specially formulated to provide stable light emission between 5 and 90+ minutes after initiating the luciferase reaction. Incorporating a 5 minute reaction-rest period ensures that light emission profiles attain maximal stability, thereby allowing assay plates to be processed in batch. By doing so, the signal output from all sample wells, from one plate to the next, may be directly compared within an experimental set.
**Preparation of Test Compounds**

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using Compound Screening Medium (CSM), as described in Step 2 of the Assay Protocol. This method avoids the adverse effects of introducing high concentrations of DMSO into the assay. The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

**NOTE:** CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup, and are considered to be ‘single-use’ reagents.

**Assay Scheme**

**Figure 1.** Assay workflow. In brief, Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user’s test compounds. Following 22 -24 hr incubation, treatment media are discarded and prepared Luciferase Detection Reagent (LDR) is added. Light emission from each assay well is quantified using a plate-reading luminometer.
**Assay Performance**

**17β-Estradiol**
- EC₅₀ = 1.3 nM
- Hill slope = 1.41
- R² = 0.9976
- at 125 nM:
  - S/B = 140
  - %CV = 4.0
  - Z’ = 0.88

**PPT**
- EC₅₀ = 24 nM
- Hill slope = 1.33
- R² = 0.9969
- at 625 nM:
  - S/B = 145
  - %CV = 8.6
  - Z’ = 0.74

**(R,R)-THC**
- EC₅₀ = 570 nM
- Hill slope = 1.99
- R² = 0.9995
- at 2,500 nM:
  - S/B = 76
  - %CV = 12
  - Z’ = 0.62

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**Figure 2. Agonist dose-response analyses of the Human ERα assay.**

Analyses of ERα Reporter Cells using 17-β-Estradiol (provided), PPT (4,4′′-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol; Tocris), and (R,R)-THC ([R,R]-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol; Tocris). Concentrated stocks of agonists were prepared in DMSO; the highest concentration of DMSO in assay wells was 0.1%. Luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 6). Signal-to-background (S/B) and Z’ values were calculated as described by Zhang, et al. (1999). Non-linear regression and EC₅₀ analyses were performed using GraphPad Prism software. Low %CV, and high S/B and Z’ scores confirm the robust performance of this ERα Assay.

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\[ Z' = 1 - \frac{3*(SD_{\text{Control}} + SD_{\text{Background}})}{(RLU_{\text{Control}} - RLU_{\text{Background}})} \]
Figure 3. Validation of ERα Assay antagonist dose-responses (a) performed in combination with INDIGO's Live Cell Multiplex Assay (b).

ERα antagonist assays were performed using Endoxifen, Tamoxifen citrate, ICI182780 and MPP dihydrochloride (all from Tocris). Assay setup and quantification of ERα activity were performed following as described in this Technical Manual. To confirm that the observed drop in RLU values resulted from receptor inhibition, not induced cell death, the relative numbers of live cells in each assay well were determined at the end of the treatment period, as described in the Technical Manual for INDIGO's Live Cell Multiplex (LCM) Assay (#LCM-01).

Each treatment contained 3.2 nM (approximating EC75) of 17β-Estradiol. Assay plates were incubated for ~23 hrs, then processed according to the LCM Assay protocol to quantify relative numbers of live cells per treatment condition. Averaged RFU values from the antagonist treatment groups were normalized to the average RFU value of "no antagonist treatment" assay wells, which corresponds to 100% Live Cells in the LCM assay. Included were assay wells treated with 3 μM staurosporine, thereby providing a cell-death 'positive control' for the LCM assay. Immediately following the LCM assay measurements, the assay plate was further processed to quantify ERα activity for each treatment condition.

Results: LCM Assays revealed no significant variance in the numbers of live cells per assay well for any of the antagonist treatments at, or below, 2.5 μM. Hence, the observed dose-dependent reductions in RLU values are due to receptor inhibition, and not treatment-induced cell death.
II. Product Components & Storage Conditions

This Human ERα Reporter Assay System contains materials to perform three distinct groups of assays in a 96-well plate format. Reagents are configured so that each group will comprise 32 assays. If desired, however, reagents may be combined to perform either 64 or 96 assays.

The individual aliquots of Reporter Cells are provided as single-use reagents. Once thawed, reporter cells can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

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.

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα Reporter Cells</td>
<td>3 x 0.60 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Cell Recovery Medium (CRM)</td>
<td>1 x 10.5 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Compound Screening Medium (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>17β-Estradiol, 100 µM (in DMSO) (reference agonist for ERα)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>Detection Substrate</td>
<td>3 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Detection Buffer</td>
<td>3 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>Plate frame</td>
<td>1</td>
<td>ambient</td>
</tr>
<tr>
<td>Snap-in, 8-well strips (white, sterile, cell-culture ready)</td>
<td>12</td>
<td>ambient</td>
</tr>
</tbody>
</table>
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**
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO\textsubscript{2} incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8- or 12-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media basins, sterile.
- sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), or deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- antagonist reference compound (optional).

**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 9-15* 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\textsubscript{50} – EC\textsubscript{85}) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This ER\textsubscript{α} Reporter Assay System kit includes a 100 µM stock solution of 17\textbeta-Estradiol, an agonist of ER\textsubscript{α} that may be used to setup antagonist-mode assays. 1 nM 17\textbeta-Estradiol typically approximates EC\textsubscript{50} in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

We find that 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 following protocol. Note that, in *Step 6*, 100 µl of treatment media is combined with 100 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.
1) Remove **Cell Recovery Medium (CRM)** and **Compound Screening Medium (CSM)** from freezer storage and thaw.
   - **CRM** should be thawed and equilibrated to 37°C using a water bath. **CRM** pre-warmed to 37°C is required in Step 3.
   - **CSM** may be thawed in a 37°C water bath.

2.) Prepare Test Compound(s) and Reference Compound stocks to be screened for Agonist or Antagonist activities.

The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

Note that, in Step 6, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl 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 schemes carefully. This assay kit provides 35 ml of **CSM**.

This ERα Reporter Assay System kit includes a 100 µM stock solution of 17β-Estradiol, a reference agonist of ERα. The following 7-point treatment series, with concentrations presented in 5-fold decrements, provides a suitable dose-response: 100000, 20000, 4000, 800, 160, 32.0, and 6.40 pM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

3.) First, retrieve the tube of **CRM** from the 37°C water bath, sanitize the outside with a 70% ethanol swab, then place it in the cell-culture hood.

   Second, retrieve **Reporter Cells** from -80°C storage. Perform a rapid thaw of the frozen cells by transferring a 3.0 ml volume of 37°C CRM into the tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 3 - 10 minutes. The resulting volume of cell suspension will be 3.6 ml.

   Third, work in the cell culture hood to carefully mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

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.

**DAY 1 Assay Protocol:** All steps must be performed using proper aseptic technique.
5.) a. **Agonist-mode assays.** Invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into each well of the 96-well Assay Plate.

b. **Antagonist-mode assays.** Invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain an homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "A word about antagonist-mode assay setup", pg. 8). Dispense 100 µl of cell suspension into each well of the 96-well Assay Plate.

   **NOTE 5.1:** 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.

   **NOTE 5.2:** Users sometimes prefer to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear 96-well assay plate, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense 100 µl of 2x-concentration treatment media (prepared as described in Step 2) into appropriate wells of the assay plate.

7.) Transfer the assay plate into a 37°C, humidified 5% CO₂ incubator for 22 - 24 hours.

   **NOTE:** Ensure a high-humidity (≥ 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 and Detection Buffer** from -80°C storage and place them in a dark refrigerator (4°C) to thaw overnight.
9.) 30 minutes before intending to quantify ERα 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 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 first assay well. Read time may be 0.5 second (500 mSec) per well, or less.

11.) Immediately before proceeding to Step 12: To read 32 assay wells, transfer the entire volume of 1 vial of Detection Buffer into 1 vial of Detection Substrate, thereby generating a 4 ml volume of Luciferase Detection Reagent (LDR). Mix gently to avoid foaming.

12.) After 22-24 hours of incubation, remove the assay plate from the incubator. Remove the plate’s lid. Remove media contents from each well.  

NOTE: Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media via a sweeping downward movement is NOT advised. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using an 8-pin manifold (e.g., Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus.

13.) Add 100 µl per well of LDR to each well of the assay plate.

14) Allow the assay plate to rest at room temperature for at least 5 minutes following the addition of LDR. Do not shake the assay plate during this period.

15) Between 5 - 90 minutes after adding LDR, place the assay plate in the luminometer and quantify luminescence.
V. Related Products

### ERα Family of Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00401-32</td>
<td>Human ERα Reporter Assay System 3x 32 assays in 96-well format</td>
</tr>
<tr>
<td>IB00401</td>
<td>Human ERα Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB00402</td>
<td>Human ERα Reporter Assay System 1x 384-well format assays</td>
</tr>
</tbody>
</table>

Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

### Panel of ER Assays

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00421-48P</td>
<td>Human ERα and ERβ Reporter Assay PANEL 48 assays each in 1x 96-well plate</td>
</tr>
</tbody>
</table>

### LIVE Cell Multiplex (LCM) Assay

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCM-01</td>
<td>Reagent volumes sufficient to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-05</td>
<td>Reagent in 5x-bulk volume to perform 480 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagent in 10x-bulk volume to perform 960 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
</tbody>
</table>

Please refer to INDIGO Biosciences website for updated product offerings.

www.indigobiosciences.com

VI. Limited Use Disclosures

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans.

“CryoMite” is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA, USA)

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

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APPENDIX 1

Example scheme for the serial dilution of 17β-Estradiol reference agonist, and the setup of an ERα dose-response assay.

For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.
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**• Considerations for Automated Dispensing •**

When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will not be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

<table>
<thead>
<tr>
<th>Stock Reagent &amp; Volume provided</th>
<th>Volume to be Dispensed (96-well plate)</th>
<th>Excess rgt. volume available for instrument dead volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reporter Cell Suspension</td>
<td>100 µl / well</td>
<td>~ 2.4 ml</td>
</tr>
<tr>
<td>(12 ml) (prepared from kit components)</td>
<td>9.6 ml / plate</td>
<td></td>
</tr>
<tr>
<td>LDR</td>
<td>100 µl / well</td>
<td>~ 2.4 ml</td>
</tr>
<tr>
<td>(12 ml) (prepared from kit components)</td>
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<td></td>
</tr>
</tbody>
</table>

**• Assay Scheme •**

*Figure 1.* Assay workflow. *In brief,* Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user’s test compounds. Following 22 -24 hr incubation, treatment media are discarded and prepared Luciferase Detection Reagent (LDR) is added. Light emission from each assay well is quantified using a plate-reading luminometer.
**Assay Performance**

![Graph showing agonist dose-response analyses of the Human ERα assay.](image)

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Figure 3. Validation of ERα Assay antagonist dose-responses (a) performed in combination with INDIGO's Live Cell Multiplex Assay (b).

ERα antagonist assays were performed using Endoxifen, Tamoxifen citrate, ICI182780 and MPP dihydrochloride (all from Tocris). Assay setup and quantification of ERα activity were performed following as described in this Technical Manual. To confirm that the observed drop in RLU values resulted from receptor inhibition, not induced cell death, the relative numbers of live cells in each assay well were determined at the end of the treatment period, as described in the Technical Manual for INDIGO's Live Cell Multiplex (LCM) Assay (#LCM-01).

Each treatment contained 3.2 nM (approximating EC_{75}) of 17β-Estradiol. Assay plates were incubated for ~23 hrs, then processed according to the LCM Assay protocol to quantify relative numbers of live cells per treatment condition. Averaged RFU values from the antagonist treatment groups were normalized to the average RFU value of "no antagonist treatment" assay wells, which corresponds to 100% Live Cells in the LCM assay. Included were assay wells treated with 3 μM staurosporine, thereby providing a cell-death 'positive control' for the LCM assay. Immediately following the LCM assay measurements, the assay plate was further processed to quantify ERα activity for each treatment condition.

Results: LCM Assays revealed no significant variance in the numbers of live cells per assay well for any of the antagonist treatments at, or below, 2.5 μM. Hence, the observed dose-dependent reductions in RLU values are due to receptor inhibition, and not treatment-induced cell death.
II. Product Components & Storage Conditions

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

The aliquot of ERα Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

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.

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>• ERα Reporter Cells</td>
<td>1 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Cell Recovery Medium (CRM)</td>
<td>1 x 10.5 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Compound Screening Medium (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• 17β-Estradiol, 100 µM (in DMSO)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>(reference agonist for ERα)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>• Detection Substrate</td>
<td>1 x 6.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Detection Buffer</td>
<td>1 x 6.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• 96-well assay plate</td>
<td>1</td>
<td>ambient</td>
</tr>
<tr>
<td>(white, sterile, cell-culture ready)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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**
- 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- or 12-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media basins, sterile.
- sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), or deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- antagonist reference compound (optional).

**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 9-15* 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 the test compound(s) to be evaluated for antagonist activity. This ER$\alpha$ Reporter Assay System kit includes a 100 $\mu$M stock solution of 17$\beta$-Estradiol, an agonist of ER$\alpha$ that may be used to setup antagonist-mode assays. 1 nM 17$\beta$-Estradiol typically approximates EC$_{50}$ in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

We find that 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 following protocol. Note that, in *Step 6*, 100 $\mu$l of treatment media is combined with 100 $\mu$l of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.
**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.
   - CRM should be thawed and equilibrated to 37°C using a water bath. CRM pre-warmed to 37°C is required in Step 3.
   - CSM may be thawed in a 37°C water bath.

2.) Prepare Test Compound(s) and Reference Compound stocks to be screened for Agonist or Antagonist activities.

   The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

   Note that, in Step 6, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl 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 schemes carefully. This assay kit provides 35 ml of CSM.

   This ERα Reporter Assay System kit includes a 100 µM stock solution of 17β-Estradiol, a reference agonist of ERα. The following 7-point treatment series, with concentrations presented in 5-fold decrements, provides a suitable dose-response: 100000, 20000, 4000, 800, 160, 32.0, and 6.40 pM, and including a 'no treatment' control. APPENDIX 1 provides an example for generating such a dilution series.

3.) First, retrieve the tube of CRM from the 37°C water bath, sanitize the outside with a 70% ethanol swab, then place it in the cell-culture hood.

   Second, retrieve Reporter Cells from -80°C storage. Perform a rapid thaw of the frozen cells by transferring a 10 ml volume of 37°C CRM into the tube of frozen cells. Recap the tube of Reporter Cells and immediately place it in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be 12 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.) **a. Agonist-mode assays.** Invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into each well of the 96-well Assay Plate.

~ or ~

**b. Antagonist-mode assays.** Invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain an homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "A word about antagonist-mode assay setup", pg. 8). Dispense 100 µl of cell suspension into each well of the 96-well Assay Plate.

**NOTE 5.1:** 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.

**NOTE 5.2:** Users sometimes prefer to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear 96-well assay plate, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense 100 µl of 2x-concentration treatment media (prepared as described in Step 2) into appropriate wells of the assay plate.

7.) Transfer the assay plate into a 37°C, humidified 5% CO\textsubscript{2} incubator for 22 - 24 hours.

**NOTE:** Ensure a high-humidity (≥ 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 and Detection Buffer from -80°C storage and place them in a dark refrigerator (4°C) to thaw overnight.
9.) 30 minutes before intending to quantify ERα 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 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 first assay well. Read time may be 0.5 second (500 mSec) per well, or less.

11.) Immediately before proceeding to Step 12, transfer the entire volume of Detection Buffer into the vial of Detection Substrate, thereby generating a 12 ml volume of 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 droplets. Cells will remain tightly adhered to well bottoms.

13.) Add 100 µl of LDR to each well of the assay plate.

14) Allow the assay plate to rest at room temperature for at least 5 minutes following the addition of LDR. Do not shake the assay plate during this period.

15) Between 5 - 90 minutes after adding LDR, place the assay plate in the luminometer and quantify luminescence.

DAY 2 Assay Protocol: Subsequent manipulations do not require special regard for aseptic technique, and may be performed on a bench top.
V. Related Products

### ERα Family of Assay Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00401-32</td>
<td>Human ERα Reporter Assay System 3x 32 assays in 96-well format</td>
</tr>
<tr>
<td>IB00401</td>
<td>Human ERα Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB00402</td>
<td>Human ERα Reporter Assay System 1x 384-well format assays</td>
</tr>
</tbody>
</table>

Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

### Panel of ER Assays

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>IB00421-48P</td>
<td>Human ERα and ERβ Reporter Assay PANEL 48 assays each in 1x 96-well plate</td>
</tr>
</tbody>
</table>

### LIVE Cell Multiplex (LCM) Assay

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
<tbody>
<tr>
<td>LCM-01</td>
<td>Reagent volumes sufficient to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-05</td>
<td>Reagent in 5x-bulk volume to perform 480 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagent in 10x-bulk volume to perform 960 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
</tbody>
</table>

Please refer to INDIGO Biosciences website for updated product offerings.

[www.indigobiosciences.com](http://www.indigobiosciences.com)

VI. Limited Use Disclosures

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans.

“CryoMite” is a Trademark™ of INDIGO Biosciences, Inc. (State College, PA, USA)

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

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APPENDIX 1
Example scheme for the serial dilution of 17β-Estradiol reference agonist, and the setup of an ERα dose-response assay.

For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.
Human Estrogen Receptor Alpha
(ERα; ESR1; NR3A1)
Reporter Assay System

384-well Format Assays
Product # IB00402

Technical Manual
(version 6.0)

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Human ERα Reporter Assay System
384-well Format Assays

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I. Description

- The Assay System -

This nuclear receptor assay system utilizes proprietary non-human cells engineered to provide constitutive, high-level expression of the full-length Human Estrogen Receptor 1 (NR3A1), a ligand-dependent transcription factor commonly referred to as ERα.

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to an ERα-responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in ERα activity.

Luciferase gene expression occurs after ligand-bound ERα 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 target 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.

ERα Reporter Cells are prepared using INDIGO’s proprietary CryoMite™ process. This cryo-preservation method yields exceptional 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, cell titer adjustments, or the pre-incubation of reporter cells prior to assay setup.

The principle application of this assay product is in the screening of test samples to quantify functional activities, either agonist or antagonist, that they may exert against the human estrogen receptor. It is an all-inclusive assay system that includes, in addition to ERα Reporter Cells, two optimized media for use during cell culture and in diluting the test samples, a reference agonist, Luciferase Detection Reagent, a cell culture-ready assay plate, and a detailed protocol.

- 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 Mg²⁺-dependent reaction that consumes O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PPi, CO₂, and photon emission. Luminescence intensity of the reaction is quantified using a luminometer, and is reported in terms of Relative Light Units (RLU’s).

INDIGO’s Nuclear Receptor Reporter Assay Systems feature a luciferase detection reagent specially formulated to provide stable light emission between 30 and 100+ minutes after initiating the luciferase reaction (see APPENDIX 2). 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.
**Preparation of Test Compounds**

Most commonly, test compounds are solvated at high-concentration in DMSO, and these are stored as master stocks. Master stocks are then diluted to appropriate working concentrations immediately prior to setting up the assay. Users are advised to dilute test compounds to 2x-concentration stocks using **Compound Screening Medium (CSM)**, as described in Step 2 of the **Assay Protocol**. This method avoids the adverse effects of introducing high concentrations of DMSO into the assay. The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

*NOTE*: CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is recommended that test compound dilutions are prepared in CSM immediately prior to assay setup, and are considered to be 'single-use' reagents.

**Considerations for Automated Dispensing**

When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will not be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

<table>
<thead>
<tr>
<th>Stock Reagent &amp; Volume provided</th>
<th>Volume to be Dispensed (384-well plate)</th>
<th>Excess rgt. volume available for instrument dead volume</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reporter Cell Suspension</strong></td>
<td>15 µl / well</td>
<td>~ 1.7 ml</td>
</tr>
<tr>
<td>7.5 ml (prepared from kit components)</td>
<td>5.8 ml / plate</td>
<td></td>
</tr>
<tr>
<td><strong>Detection Substrate</strong></td>
<td>15 µl / well</td>
<td>~ 2 ml</td>
</tr>
<tr>
<td>7.8 ml</td>
<td>5.8 ml / plate</td>
<td></td>
</tr>
</tbody>
</table>

**Assay Scheme**

*Figure 1.* Assay workflow. *In brief,* Reporter Cells are dispensed into wells of the assay plate and then immediately dosed with the user’s test compounds. Following 22 - 24 hr incubation, Detection Reagent is added and light emission from each assay well is quantified using a plate-reading luminometer.
**Assay Performance**

![Graph showing dose-response curves for 17β-Estradiol, PPT, and (R,R)-THC](image)

- **17β-Estradiol**
  - EC\textsubscript{50} = 1.3 nM
  - Hill slope = 1.41
  - R\textsuperscript{2} = 0.9976
  - at 125 nM:
    - S/B = 140
    - %CV = 4.0
    - Z' = 0.88

- **PPT**
  - EC\textsubscript{50} = 24 nM
  - Hill slope = 1.33
  - R\textsuperscript{2} = 0.9969
  - at 625 nM:
    - S/B = 145
    - %CV = 8.6
    - Z' = 0.74

- **(R,R)-THC**
  - EC\textsubscript{50} = 570 nM
  - Hill slope = 1.99
  - R\textsuperscript{2} = 0.9995
  - at 2,500 nM:
    - S/B = 76
    - %CV = 12
    - Z' = 0.62

---

**Figure 2. Agonist dose-response analyses of the Human ERα assay.**

Analyses of ERα Reporter Cells using 17β-Estradiol (provided), PPT (4,4'4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol; Tocris), and (R,R)-THC ([R,R]-5,11-diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol; Tocris). Concentrated stocks of agonists were prepared in DMSO; the highest concentration of DMSO in assay wells was 0.1%. Luminescence was quantified using a GloMax-Multi+ luminometer (Promega). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n ≥ 6). Signal-to-background (S/B) and Z’ values were calculated as described by Zhang, et al. (1999)\(^1\). Non-linear regression and EC\textsubscript{50} analyses were performed using GraphPad Prism software. Low %CV, and high S/B and Z’ scores confirm the robust performance of this ERα Assay.


\[Z' = 1 - \left[3 \times (SD_{\text{Control}} + SD_{\text{Background}}) / (RLU_{\text{Control}} - RLU_{\text{Background}})\right]\]
Figure 3. Validation of ERα Assay antagonist dose-responses (a) performed in combination with INDIGO's Live Cell Multiplex Assay (b).

ERα antagonist assays were performed using Endoxifen, Tamoxifen citrate, ICI182780 and MPP dihydrochloride (all from Tocris). Assay setup and quantification of ERα activity were performed following as described in this Technical Manual. To confirm that the observed drop in RLU values resulted from receptor inhibition, not induced cell death, the relative numbers of live cells in each assay well were determined at the end of the treatment period, as described in the Technical Manual for INDIGO's Live Cell Multiplex (LCM) Assay (#LCM-01).

Each treatment contained 3.2 nM (approximating EC75) of 17β-Estradiol. Assay plates were incubated for ~23 hrs, then processed according to the LCM Assay protocol to quantify relative numbers of live cells per treatment condition. Averaged RFU values from the antagonist treatment groups were normalized to the average RFU value of "no antagonist treatment" assay wells, which corresponds to 100% Live Cells in the LCM assay. Included were assay wells treated with 3 µM staurosporine, thereby providing a cell-death 'positive control' for the LCM assay. Immediately following the LCM assay measurements, the assay plate was further processed to quantify ERα activity for each treatment condition.

Results: LCM Assays revealed no significant variance in the numbers of live cells per assay well for any of the antagonist treatments at, or below, 2.5 µM. Hence, the observed dose-dependent reductions in RLU values are due to receptor inhibition, and not treatment-induced cell death.
II. Product Components & Storage Conditions

This Human ERα Reporter Assay System contains materials to perform assays in a single 384-well assay plate.

The aliquot of ERα Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen or maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

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.

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>• ERα Reporter Cells</td>
<td>1 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Cell Recovery Medium (CRM)</td>
<td>1 x 6 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Compound Screening Medium (CSM)</td>
<td>1 x 35 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• 17β-Estradiol, 100 µM (in DMSO) (reference agonist for ERα)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Detection Substrate</td>
<td>1 x 7.8 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• 384-well assay plate (white, sterile, cell-culture ready)</td>
<td>1</td>
<td>ambient</td>
</tr>
</tbody>
</table>
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**
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO₂ incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8- or 12-channel electronic, repeat-dispensing pipettes & sterile tips
- disposable media basins, sterile.
- sterile multi-channel media basins (such as the Heathrow Scientific "Dual-Function Solution Basin"), or deep-well plates, or appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
- antagonist reference compound (optional).

**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 9-15 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 EC₅₀ – EC₈₅) of a known agonist AND the test compound(s) to be evaluated for antagonist activity. This ERα Reporter Assay System kit includes a 100 µM stock solution of **17β-Estradiol**, an agonist of ERα that may be used to setup antagonist-mode assays. 1 nM 17β-Estradiol typically approximates EC₅₀ in this reporter assay. Hence, it presents a reasonable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

We find that 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 following protocol. Note that, in **Step 6**, 15 µl of treatment media is combined with 15 µl of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x-concentration of the reference agonist. **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a desired 2x-concentration of agonist.
**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.
   - CRM should be thawed and equilibrated to **37°C** using a water bath. CRM pre-warmed to 37°C is required in **Step 3**.
   - CSM may be thawed in a **37°C** water bath.

2.) **Prepare Test Compound(s) and Reference Compound stocks to be screened for Agonist or Antagonist activities.**

   The final concentration of total DMSO carried over into assay reactions should never exceed 0.4%.

   Note that, in **Step 6**, 15 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 15 µl 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 schemes carefully. This assay kit provides 35 ml of CSM.

   This ERα Reporter Assay System kit includes a 100 µM stock solution of **17β-Estradiol**, a reference agonist of ERα. The following 7-point treatment series, with concentrations presented in 5-fold decrements, provides a suitable dose-response: 100000, 20000, 4000, 800, 160, 32.0, and 6.40 pM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

3.) **First**, retrieve the tube of CRM from the 37°C water bath, sanitize the outside with a 70% ethanol swab, then place it in the cell-culture hood.

   **Second**, retrieve **Reporter Cells** from -80°C storage. Perform a rapid thaw of the frozen cells by transferring a 5.5 ml volume of 37°C CRM into the tube of frozen cells. Recap the tube of Reporter Cells and immediately 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.) **a. Agonist-mode assays.** Invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Without delay, dispense 15 µl of cell suspension into each well of the 384-well Assay Plate.

~ or ~

**b. Antagonist-mode assays.** Invert the tube of Reporter Cells several times to disperse any cell aggregates, and to gain an homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of reference agonist (refer to "A word about antagonist-mode assay setup", pg. 8). Dispense 15 µl of cell suspension into each well of the 384-well Assay Plate.

**NOTE 5.1:** 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.

**NOTE 5.2:** Users sometimes prefer to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed into a clear 384-well assay plate, treated +/- test compounds as desired, and incubated overnight in identical manner to those reporter cells contained in the white assay plate.

6.) Dispense 15 µl of 2x-concentration treatment media (prepared as described in Step 2) into appropriate wells of the assay plate.

7.) Transfer the assay plate into a 37°C, humidified 5% CO₂ incubator for 22 - 24 hours.

**NOTE:** Ensure a high-humidity (≥ 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 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.) 30 minutes before intending to quantify ERα activity, remove Detection Substrate from the refrigerator and place them in a low-light area so that it may equilibrate to room temperature. Gently invert the tube several times to ensure an homogenous solution.

   NOTE: Do NOT actively warm Detection Substrate above room temperature. If these solutions were not allowed to thaw overnight at 4°C, a room temperature water bath may be used to expedite thawing.

10.) Turn on the luminometer. Set the instrument to perform a single 5 second “plate shake” prior to reading the first assay well. Read time may be 0.5 second (500 mSec) per well, or less.

11.) Following 22 - 24 hours of incubation, retrieve the assay plate from the incubator. Remove the plate’s lid. Add 15 µl 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.

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

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

13.) Place the assay plate in the luminometer and quantify luminescence.
V. Related Products

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<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
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<tbody>
<tr>
<td>IB00401-32</td>
<td>Human ERα Reporter Assay System 3x 32 assays in 96-well format</td>
</tr>
<tr>
<td>IB00401</td>
<td>Human ERα Reporter Assay System 1x 96-well format assay</td>
</tr>
<tr>
<td>IB00402</td>
<td>Human ERα Reporter Assay System 1x 384-well format assays</td>
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</table>

Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.

Panel of ER Assays

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<th>Product No.</th>
<th>Product Description</th>
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<tr>
<td>IB00421-48P</td>
<td>Human ERα and ERβ Reporter Assay PANEL 48 assays each in 1x 96-well plate</td>
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LIVE Cell Multiplex (LCM) Assay

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<th>Product Descriptions</th>
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<tr>
<td>LCM-01</td>
<td>Reagent volumes sufficient to perform 96 Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-05</td>
<td>Reagent in 5x-bulk volume to perform 480 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
</tr>
<tr>
<td>LCM-10</td>
<td>Reagent in 10x-bulk volume to perform 960 Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats</td>
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Please refer to INDIGO Biosciences website for updated product offerings.

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APPENDIX 1

Example scheme for the serial dilution of 17β-Estradiol reference agonist, and the setup of an ERα 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.
APPENDIX 2
Signal Stability of the Nuclear Receptor Reporter Assay

The Human PPARα 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 3, 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 ≥ 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 3. Stability of the luminescence signal from 384-well format, homogenous assay protocol. PPARα 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 photon emission over 500 mSec. Average RLU values were calculated, then normalized so that the luminescence signal at 30 minutes = 100%.