

**Human Estrogen-Related Receptor, Gamma  
(NR3B3, ERR $\gamma$ )  
Reporter Assay System**

**3x 32 Assays in 96-well Format**  
Product # IB08021-32

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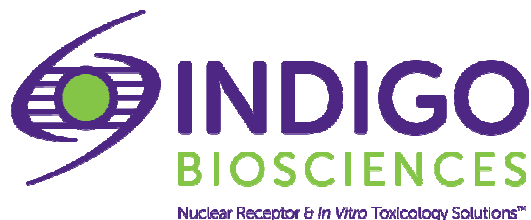
**Technical Manual**  
*(version 7.1i)*

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## Human ERR $\gamma$ 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 mammalian cells engineered to provide high-level expression of a hybrid form of the **Human Estrogen-Related Receptor Gamma (NR3B3)**. The N-terminal DNA binding domains (DBD) of the native ERR $\gamma$  has been substituted with that of the yeast GAL4-DBD. The reporter gene is beetle luciferase functionally linked to the GAL4 upstream activation sequence (UAS).

As is true *in vivo*, these reporter cells express ERR $\gamma$  that is constitutively active in the (putative) absence of a ligand binding event. Interestingly, the ligand binding domain of ERR $\gamma$  may be occupied by a ligand that further elevates the constitutive activity of the receptor (an agonist response), or one that results in a dose-dependent loss of constitutive activity (an inverse-agonist response)<sup>1</sup>. **Figure 2** demonstrates the high constitutive activity of ERR $\gamma$  in the absence of added ligand, as well as agonist and inverse-agonist responses of the receptor upon ligand binding. Therefore, the principal application of this assay system is in the screening of test samples to quantify either *agonist* or *inverse-agonist* activities that they may exert against human ERR $\gamma$ .

ERR $\gamma$  Reporter Cells are prepared using INDIGO's proprietary **CryoMite™** process. This cryo-preservation method yields high cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for cumbersome intermediate treatment steps such as spin-and-rinse of cells, viability determinations, or cell titer adjustments prior to assay setup.

INDIGO Bioscience's assays are all-inclusive cell-based assay systems. In addition to ERR $\gamma$  Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, the reference inverse-agonist 4-Hydroxy tamoxifen, Luciferase Detection Reagent, and a cell culture-ready assay plate.

### ▪ The Assay Chemistry ▪

INDIGO's cell-based assays capitalize on the extremely low background, high-sensitivity, and broad linear dynamic range of bio-luminescence reporter gene technology.

Reporter Cells incorporate the cDNA encoding beetle luciferase, a 62 kD protein originating from the North American firefly (*Photinus pyralis*). Luciferase catalyzes the mono-oxidation of D-luciferin in a Mg<sup>+2</sup>-dependent reaction that consumes O<sub>2</sub> and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP<sub>i</sub>, CO<sub>2</sub>, 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 assay kits 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 ▪

Small molecule test compounds are typically solvated in DMSO at high concentrations; ideally 1,000x-concentrated stocks relative to the highest desired treatment concentration in the assay. Using high-concentration stocks minimizes DMSO carry-over into the assay plates. Immediately prior to setting up an assay, the master stocks are serially diluted using one of two alternative strategies:

1.) As described in *Step 2*, and depicted in Appendix 1 for the reference inverse-agonist, **Compound Screening Medium (CSM)** may be used as the diluent to make serial dilutions of test compounds to achieve the desired final assay concentration series.

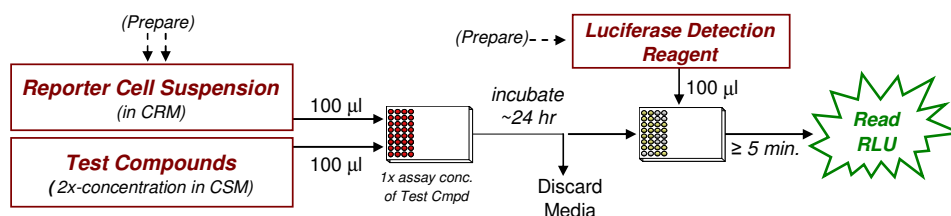
Alternatively, if test compound solubility is expected to be problematic, 2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared DMSO dilution series.

Regardless of the dilution method used, the final concentration of total DMSO carried over into assay wells should not exceed 0.4%. Significant DMSO-induced cytotoxicity can be expected above 0.4%.

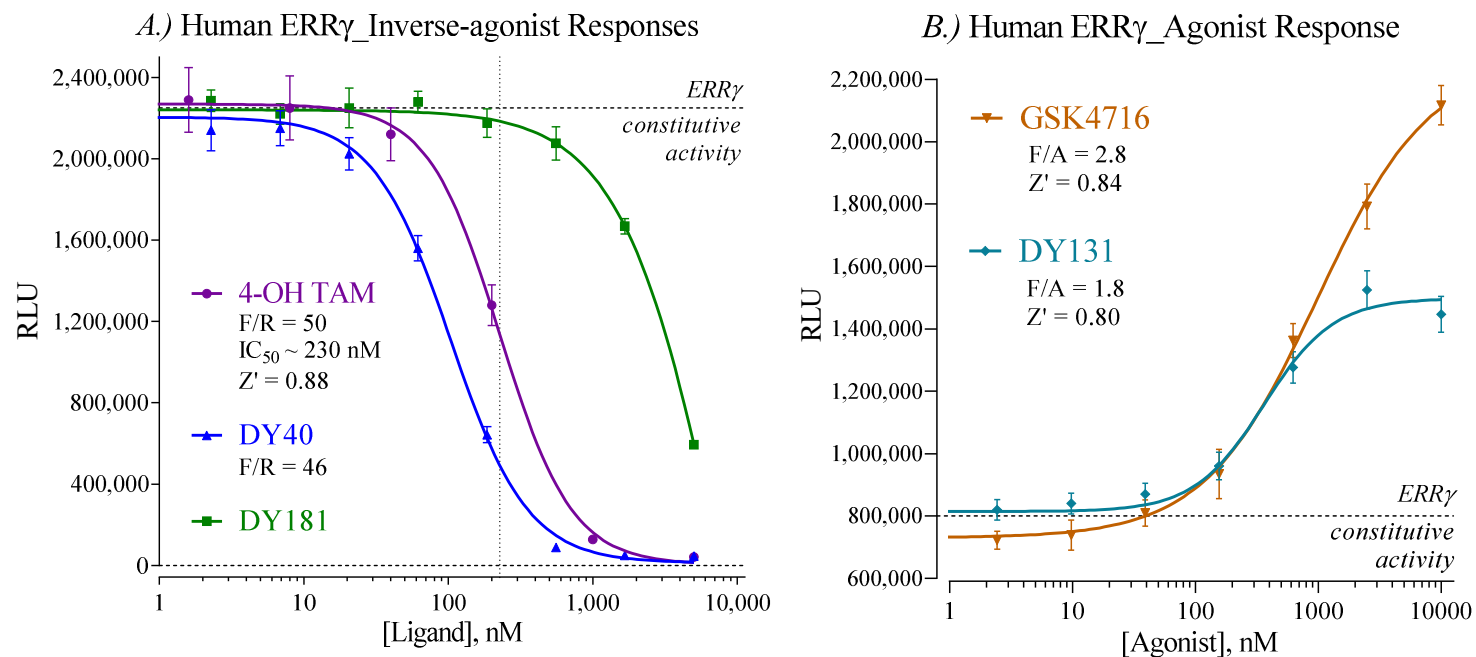
*NOTE:* CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of extremely hydrophobic test compounds diluted in CSM may lack long-term stability and/or solubility, especially if further stored at low temperatures. 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*, 100  $\mu$ l/well of Reporter Cells are dispensed into wells of the assay plate, followed immediately by dispensing 100  $\mu$ l/well of the prepared treatment media. Following 22 - 24 hours incubation, treatment media are discarded, and prepared Luciferase Detection Reagent (LDR) is added. The intensity of light emission (in units of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



▪ Assay Performance ▪



**Figure 2. Dose-response analyses of Human ERR $\gamma$ .** ERR $\gamma$  Assays were performed using A.) the reference agonists GSK4716<sup>1</sup> and DY131<sup>2</sup> (Tocris), and B.) the inverse-agonists 4-Hydroxy Tamoxifen (provided), DY40<sup>4</sup> and DY181<sup>4</sup>. INDIGO's Live Cell Multiplex assay confirmed that none of the treatment concentrations induced cytotoxicity (data not shown). Averaged relative light units (RLU) and their corresponding values of standard deviation and percent coefficient of variation were determined for each treatment concentration (n = 3). Values of fold-activation (F/A) and fold-reduction (F/R) in ERR $\gamma$  activities were calculated by normalizing respective RLU values from test compound-treated reporter cells to the RLU value of untreated reporter cells. Z' values were calculated as described by Zhang, *et al.* (1999)<sup>5</sup>. Non-linear regression and respective EC<sub>50</sub> and IC<sub>50</sub> determination were performed using GraphPad Prism software.

## II. Product Components & Storage Conditions

This Human ERR $\gamma$  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.

**Reporter cells are temperature sensitive! To ensure maximal viability the tube of cells must be maintained at -80°C until immediately prior to the rapid-thaw procedure described in Step 3 of this protocol.**

Assay kits are shipped on dry ice. Upon receipt of the kit transfer it to -80°C storage. If you wish to first inspect and inventory the individual kit components be sure to first transfer and submerge the tube of cells in dry ice.

The aliquot of Reporter Cells is provided as a single-use reagent. Once thawed, reporter cells can NOT be refrozen, nor can they be maintained in extended culture with any hope of retaining downstream assay performance. Therefore, extra volumes of these reagents should be discarded after assay setup.

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

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ ERR $\gamma$ Reporter Cells	3 x 0.60 mL	-80°C
▪ Cell Recovery Medium (CRM)	1 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ 4-Hydroxy Tamoxifen, 10 mM (in DMSO) (reference inverse-agonist for ERR $\gamma$ )	1 x 30 $\mu$ L	-20°C
▪ Detection Substrate	3 x 2.0 mL	-80°C
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate frame	1	ambient
▪ Snap-in, 8-well strips (white, sterile, cell-culture ready)	12	-20°C

## III. Materials to be Supplied by the User

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

### DAY 1

- dry ice bucket (*Step 3*)
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO<sub>2</sub> incubator for mammalian cell culture.
- 37°C water bath.
- 70% alcohol wipes
- 8-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* sterilized 96 deep-well blocks (*e.g.*, Axygen Scientific, #P-2ML-SQ-C-S), *or* appropriate similar vessel for generating dilution series of reference and test compound(s).
- *Optional*: clear 96-well assay plate, sterile, cell culture treated, for viewing cells on *Day 2*.

**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.

**DAY 1 Assay Protocol:** All steps must be performed using aseptic technique.

- 1.) Remove **Cell Recovery Medium (CRM)** and **Compound Screening Medium (CSM)** from freezer storage, thaw and equilibrate to 37°C using a water bath.
- 2.) **Prepare dilutions of treatment compounds** (first see *Note 5.4*): Prepare Test Compound treatment media.

Total DMSO carried over into assay reactions should not 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. Manage dilution volumes carefully. This assay kit provides 35 ml of CSM.

**Preparing the positive control:** This ERRγ Reporter Assay System kit includes a 10 mM stock solution of 4-hydroxy-tamoxifen, an inverse-agonist of ERRγ. The following 7-point treatment series, with concentrations presented in 5-fold decrements, provides a complete inverse-agonist dose-response: 5000, 1000, 200, 40.0, 8.00, 1.60, and 0.320 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

- 3.) **Rapid Thaw of the Reporter Cells:** *First*, retrieve the tube of **CRM** from the 37°C water bath and sanitize the outside with a 70% ethanol swab.

*Second*, retrieve **Reporter Cells** from -80°C storage: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, and 3 tubes for 96 assay wells. Place the tube(s) directly into dry ice and transport the cells to the laminar flow hood. When ready to begin, place the tube(s) of reporter cells into a rack and, *without delay*, perform a rapid thaw of the frozen cells by transferring a 3.0 ml volume of 37°C CRM into each tube of frozen cells. Recap the tube(s) and immediately place it in a 37°C water bath for 5 - 10 minutes. If only one tube of reporter cells is thawed (32 assays), the resulting volume of cell suspension will be 3.6 ml.

*Third*, during the 5 - 10 minutes incubation period, 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 and sanitize the outside surface of the tube with a 70% alcohol swab.

- 5.) Gently invert the tube of Reporter Cells several times to gain a homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into each well of the assay plate.

*NOTE 5.1:* If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'cell blank' wells (meaning cell-free but containing 'Compound Screening Media') must be included in the assay plate to allow quantification of plate-specific fluorescence background (refer to the LCMA Technical Manual).

(continued ....)

*NOTE 5.2:* 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 5.3:* Users sometimes wish to examine the reporter cells using a microscope. If so, the extra volume of cell suspension provided with each kit may be dispensed (100  $\mu$ l/well) into a clear 96-well cell culture treated assay plate, followed by 100  $\mu$ l/well of CSM. Incubated overnight in identical manner to those reporter cells contained in the white assay plate.

*NOTE 5.4:* For logistical reasons, some users find it more convenient to first plate the reporter cells and then prepare their test compound dilutions. That strategy works equally well. Once plated, cells may be placed in an incubator for up to 3 hours before proceeding to *Step 6*.

6.) Dispense 100  $\mu$ l of 2x-concentration treatment media into appropriate assay wells.

7.) Transfer the assay plate into a cell culture incubator (37°C, humidified 5% CO<sub>2</sub>) for 22 - 24 hours.

*NOTE:* Ensure a high-humidity ( $\geq 70\%$ ) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

8.) For greater convenience on *Day 2*, retrieve the appropriate number of sets of **Detection Substrate and Detection Buffer** (one set for each vial of reporter cells used) from freezer storage and place them 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 receptor activity, remove **Detection Substrate** from the refrigerator and place them in a low-light area so that it may equilibrate to room temperature.

*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.) Set the plate-reader to "luminescence" mode. Set the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Read time may be set to 0.5 second (500 mSec) per well, *or less*.

11.) *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 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 is NOT advised. Do *not* touch the well bottom or run the tip of the aspiration device around the bottom circumference of the assay well. Such practices will result in destruction of the reporter cells and greatly increased well-to-well variability. Complete removal of the media is efficiently performed by tilting the plate on edge and aspirating media using a single tip or an 8-pin manifold (*e.g.*, Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus.

13.) Add 100  $\mu$ l of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for 5 - 10 minutes. Do not shake the assay plate during this period.

14.) Quantify luminescence.



## V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
<b>ERR<math>\gamma</math> Assay Products</b>	
IB08021-32	Human ERR $\gamma$ Reporter Assay System 3x 32 assays in 96-well format
IB08021	Human ERR $\gamma$ Reporter Assay System 1x 96-well format assay
IB08022	Human ERR $\gamma$ Reporter Assay System 1x 384-well format assays
Bulk volumes of assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	
<b>LIVE Cell Multiplex (LCM) Assay</b>	
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 <b>5x bulk volume</b> to perform <b>480</b> Live Cell Assays performed in 5 x 96-well assay plates
LCM-10	Reagent in <b>10x bulk volume</b> to perform <b>960</b> Live Cell Assays performed in 10 x 96-well assay plates
<b>INDIGlo Luciferase Detection Reagent</b>	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents in 10 mL, 25 mL, 50 mL, and 500 mL volumes

Please refer to INDIGO Biosciences' website for updated product offerings.

[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, diagnostic, or contact use in humans or animals.

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

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## VII. Citations

<sup>1</sup> Wang L, *et al.* (2006) X-ray crystal structures of the estrogen-related receptor  $\gamma$  ligand binding domain in three function states reveal the molecular basis of small molecule regulation. *JBC*:**281** (49), 37773 - 37781.

<sup>2</sup> Yu DD and Barry MF (2005) Identification of an agonist ligand for estrogen-related receptors ERR $\beta/\gamma$ . *Bioorgan. & Med. Chem. Letters*:**15**, 1311-1313.

<sup>3</sup> Zuercher WJ, *et al.* (2005) Identification and structure-activity relationship of phenolic acyl hydrazonees as selective agonists for the estrogen-related orphan nuclear receptors ERR $\beta$  and ERR $\gamma$ . *J. Med. Chem.*:**48**, 3107-3109.

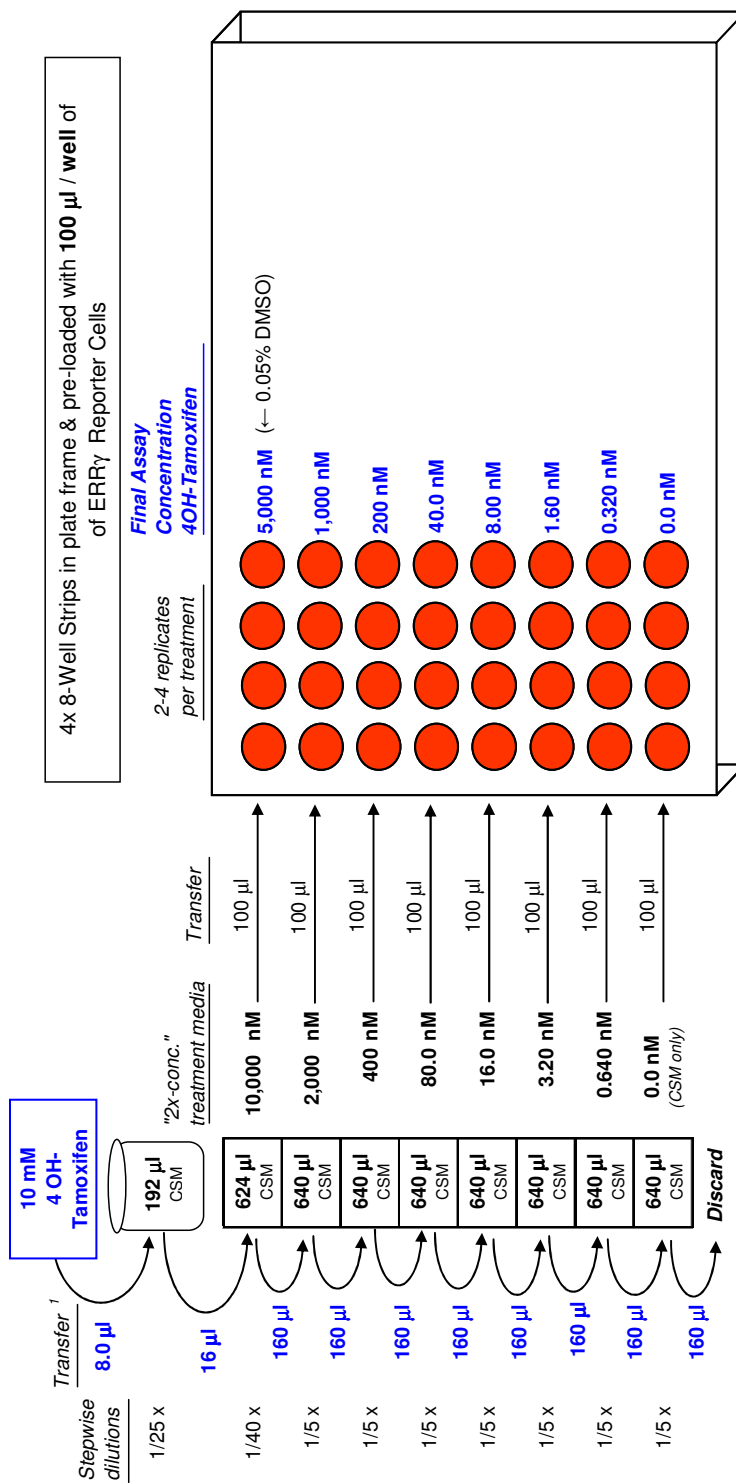
<sup>4</sup> Yu DD, Huss JM, Li H, Forman BM (2017) Identification of novel inverse agonists of estrogen-related receptors ERR $\gamma$  and ERR $\beta$ . *Bioorganic & Medicinal Chem.*:**25**, 1585 – 1599.

<sup>5</sup> Zhang JH, *et al.* (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen.*:**4**(2), 67-73.

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

## APPENDIX 1

Example scheme for the serial dilution of 4OH-Tamoxifen inverse-agonist, and the setup of an ERR $\gamma$  dose-response assay.



<sup>1</sup> For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.