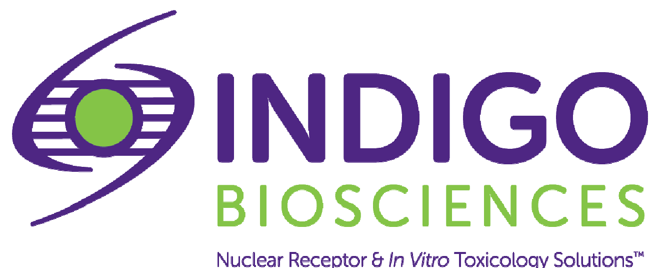

Human Estrogen-Related Receptor, Gamma
(NR3B3, ERRγ)
Reporter Assay System

96-well Format Assays
Product # IB08021

▪

Technical Manual
(version 7.1i)

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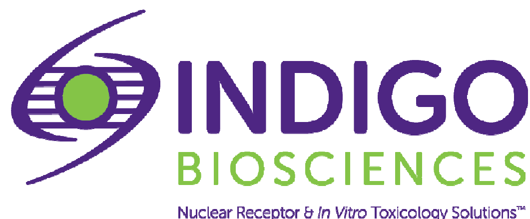

Human ERRγ Reporter Assay System
96-well Format Assays

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I. Description

▪ The Assay System ▪

This nuclear receptor assay 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 ERRγ 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 ERRγ that is constitutively active in the (putative) absence of a ligand binding event. Interestingly, the ligand binding domain of ERRγ 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 active (an inverse-agonist response)[1]. **Figure 2** demonstrates the high constitutive activity of ERRγ 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 is in the screening of test samples to quantify either *agonist* or *inverse-agonist* activities that they may exert against human ERRγ.

ERRγ 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 ERRγ 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^{+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 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.

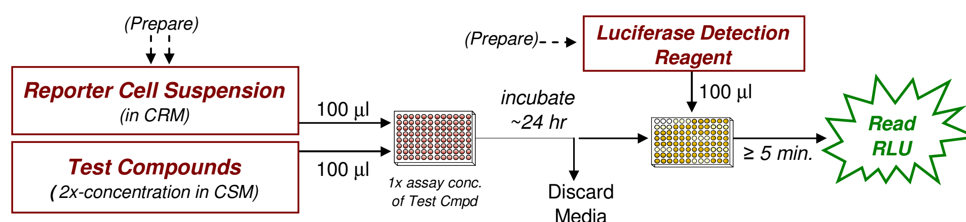
▪ Considerations for Automated Dispensing ▪

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. 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.

Stock Reagent & Volume provided	**Volume to be Dispensed** *(96-well plate)*	**Excess rgt. volume available for instrument dead volume**
Reporter Cell Suspension 12 ml *(prepared from kit components)*	100 µl / well 9.6 ml / plate	~ 2.4 ml
LDR 12 ml *(prepared from kit components)*	100 µl / well 9.6 ml / plate	~ 2.4 ml

▪ Assay Scheme ▪

Figure 1. Assay workflow. *In brief*, 100 µl/well of Reporter Cells are dispensed into wells of the assay plate, followed immediately by dispensing 100 µl/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.



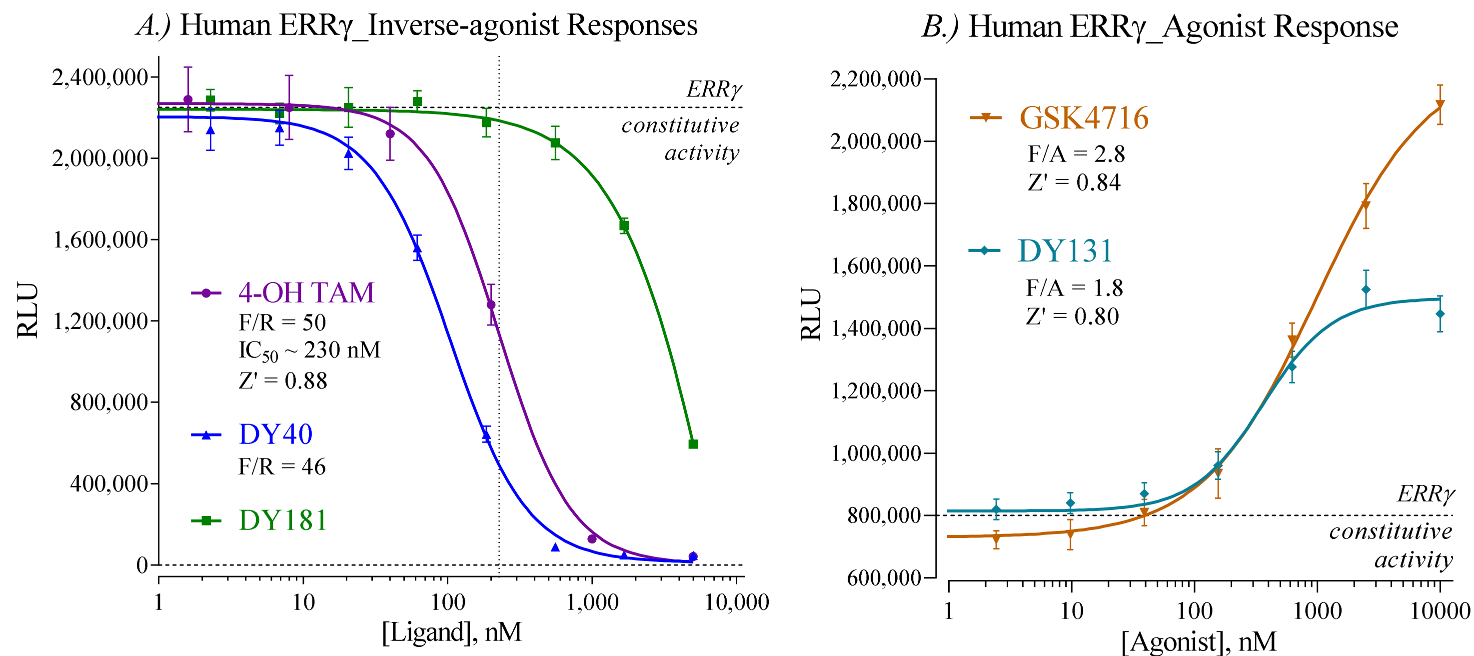


Figure 2. Dose-response analyses of Human ERRγ. ERRγ Assays were performed using *A.)* the reference agonists GSK4716[1] and DY131[2] (Tocris), and *B.)* the inverse-agonists 4-Hydroxy Tamoxifen (provided), DY40[4] and DY181[4]. INDIGO's Live Cell Multiplex assay confirmed that none of the treatment 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 ERRγ 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)[5]. Non-linear regression and respective EC_{50} and IC_{50} determination were performed using GraphPad Prism software.

II. Product Components & Storage Conditions

This ERRγ assay kit contains materials to perform assays in a single 96-well assay plate.

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.

Kit Components	*Amount*	*Storage Temp.*
▪ ERRγ Reporter Cells	1 x 2.0 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 ERRγ)	1 x 30 μL	-20°C
▪ Detection Substrate	1 x 6.0 mL	**-80°C**
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well assay plate (white, sterile, cell-culture ready)	1	ambient

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_2 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 9-14* 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 and thaw in a 37°C water bath.

2.) Prepare dilutions of treatment compounds (first see *Note 5.3*): 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). Plan dilution volumes carefully; this kit provides 35 ml of CSM.

Preparing the positive control: This ERRγ assay 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 the tube of **Reporter Cells** from -80°C storage, place it directly into a dry ice bucket and transport the cells to the laminar flow hood. When ready, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the cells by transferring 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 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, transfer the cell suspension into a reservoir and, using an 8-chanel pipette, 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).
>
> *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 µl/well) into a clear 96-well cell culture treated assay plate, followed by 100 µl/well of CSM. Incubated overnight in identical manner to those reporter cells contained in the white assay plate.
>
> *(continued ….)*

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*.

NOTE 5.5: If well-to-well variation due to 'edge-effects' is a concern this problem *may* be mitigated by dispensing sterile liquid into the *inter-well* spaces of the assay plate. Simply remove 1 tip from the 8-chanel dispenser and dispense 100 µl of sterile water into each of the seven inter-well spaces per column of wells.

6.) Dispense 100 µl of 2x-concentration treatment media into appropriate assay wells.

7.) Transfer the assay plate into a cell culture incubator (37°C, humidified 5% CO_2) for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥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 *Day 2*, retrieve **Detection Substrate** *and* **Detection Buffer** 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*, 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 discard all media contents by manually ejecting it into an appropriate waste container. *Gently* tap the inverted plate onto a clean absorbent paper towel to remove residual droplets.

13.) Add 100 µl 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

Product No.	*Product Descriptions*
ERRγ Assay Products	
IB08021-32	Human ERRγ Reporter Assay System 3x 32 assays in 96-well format
IB08021	Human ERRγ Reporter Assay System 1x 96-well format assay
IB08022	Human ERRγ 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.	

LIVE Cell Multiplex (LCM) Assay	
LCM-01	Reagent volumes sufficient to perform **96** Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in **5x bulk volume** to perform **480** Live Cell Assays performed in 5 x 96-well assay plates
LCM-10	Reagent in **10x bulk volume** to perform **960** Live Cell Assays performed in 10 x 96-well assay plates
INDIGlo Luciferase Detection Reagent	
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

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

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

[2] Yu DD and Barry MF (2005) Identification of an agonist ligand for estrogen-related receptors ERRβ/γ. Bioorgan. & Med. Chem. Letters:**15**, 1311-1313.

[3] Zuercher WJ, *et al*. (2005) Identification and structure-activity relationship of phenolic acyl hydrazaones as selective agonists for the estrogen-related orphan nuclear receptors ERRβ and ERRγ. J. Med. Chem.:**48**, 3107-3109.

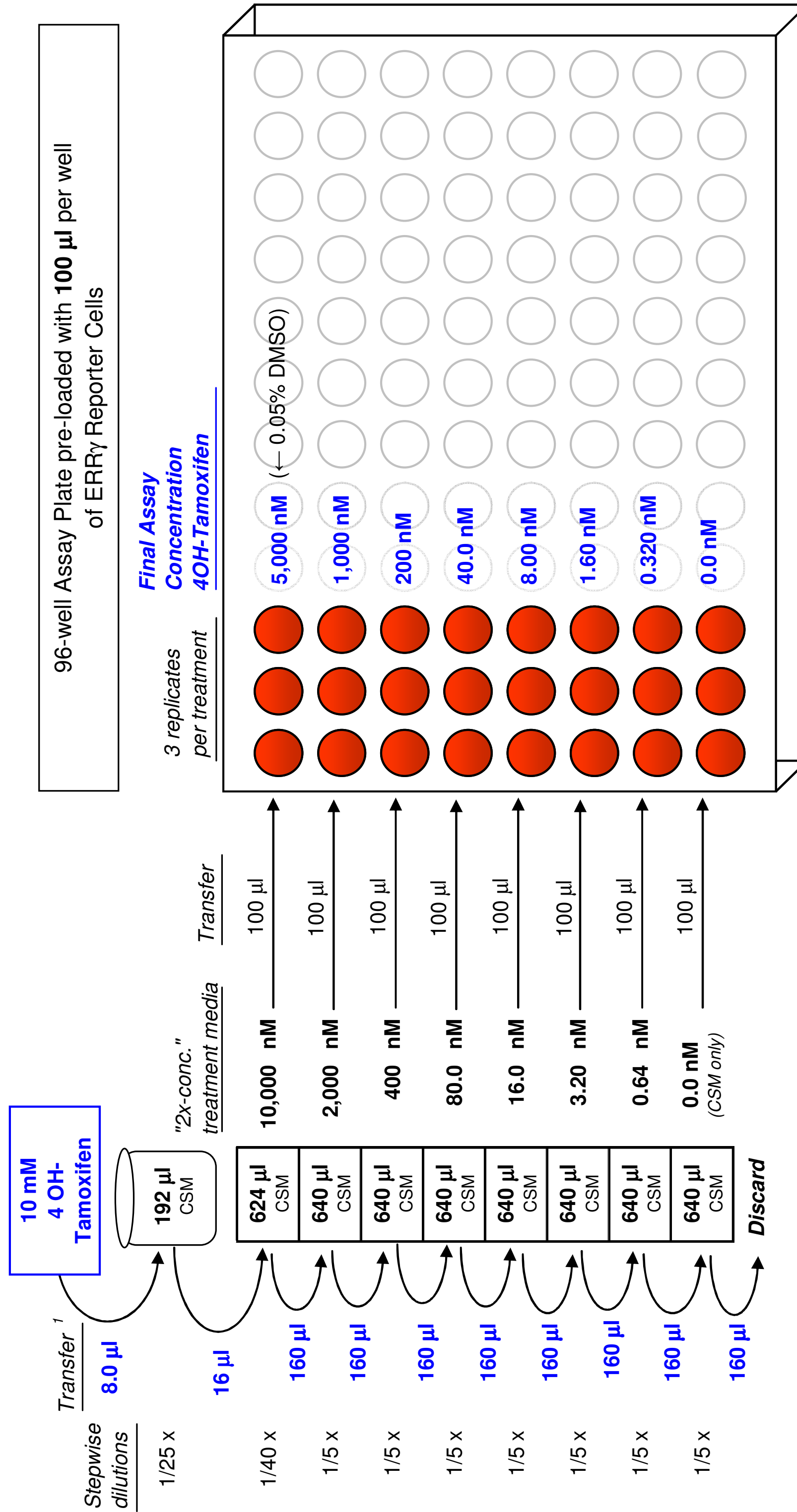
[4] Yu DD, Huss JM, Li H, Forman BM (2017) Identification of novel inverse agonists of estrogen-related receptors ERRγ and ERRβ. Bioorganic & Medicinal Chem.:**25**, 1585 – 1599.

[5] 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.

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

APPENDIX 1

Example scheme for the serial dilution of 4OH-Tamoxifen inverse-agonist, and the setup of an ERRγ 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.