

**Human Estrogen Receptor Beta
(ER β ; ESR2; NR3A2)
Reporter Assay System**

96-well Format Assays
Product # IB00411

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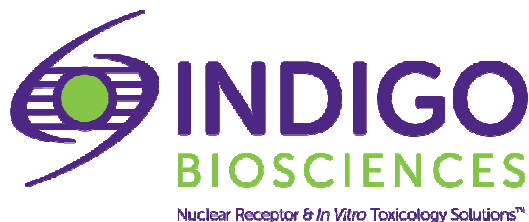
Technical Manual
(version 7.1i)

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Human ER β Reporter Assay System 96-well Format Assays

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I. Description

▪ The Assay System ▪

This nuclear receptor assay utilizes proprietary non-human cells engineered to provide constitutive, high-level expression of the full-length **Human Estrogen Receptor 2 (NR3A2)**, a ligand-dependent transcription factor commonly referred to as **ER β** .

INDIGO's Reporter Cells include the luciferase reporter gene functionally linked to a ER β -responsive promoter. Thus, quantifying changes in luciferase expression in the treated reporter cells provides a sensitive surrogate measure of the changes in ER β activity. The principal application of this reporter assay is in the screening of test samples to quantify any functional activity, either agonist or antagonist, that they may exert against human ER β .

ER β 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.

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

▪ The Assay Chemistry ▪

INDIGO's nuclear receptor reporter 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 O₂ and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP_i, 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 Assays 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 7* and depicted in Appendix 1 for the reference agonist, **Compound Screening Medium (CSM)** is used as the diluent to make serial dilutions of test compounds to achieve the desired 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 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 then are considered to be 'single-use' reagents.

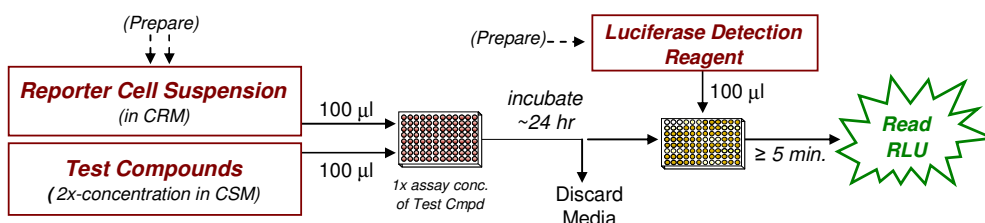
▪ Considerations for Automated Dispensing ▪

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*, 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 ▪

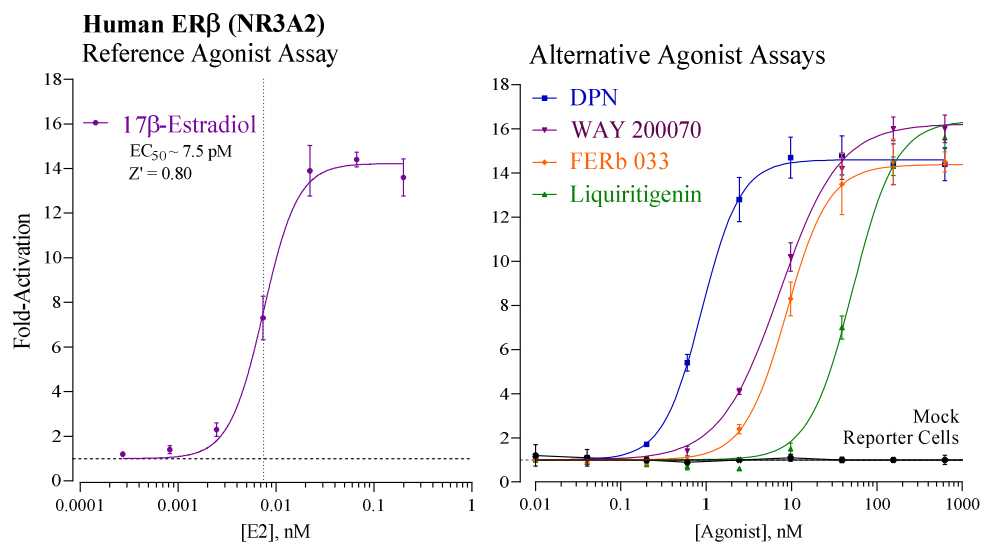


Figure 2. Activity dose-response of ERβ using various reference agonists.

Dose-response analyses of ERβ were performed according to the protocol provided in this Technical Manual. Reporter Cells were treated with 17β-Estradiol (provided), DPN, Linquiritigenin, WAY 200070, and FERb 033 (all from Tocris). Average relative light units (RLU) and corresponding standard deviation (SD) values were determined for each treatment concentration (n = 4). Values of Fold-Activation (Signal / Background) and Z' were calculated as described by Zhang, *et al.* (1999)¹. Non-linear regression and EC₅₀ analyses were performed using GraphPad Prism software.

RESULTS: EC₅₀ Z' values for 17β-estradiol confirm the robust performance of this ERβ Assay and demonstrate its suitability for use in HTS applications.¹

¹ Zhang JH, Chung TD, Oldenburg KR. (1999) A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J Biomol Screen.*:4(2), 67-73.

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

Human ER β Antagonist Assays

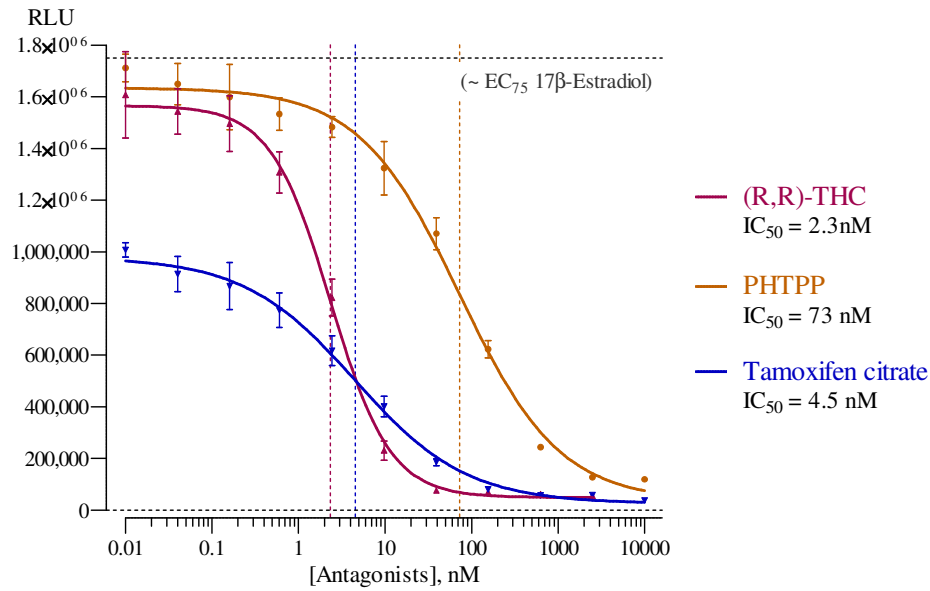


Figure 3. Human ER β antagonist-mode Assays

ER β antagonist assays were performed using Tamoxifen citrate, PHTPP and (R,R)-THC (all from Tocris). ER β Assay setup and quantification of antagonist activities were performed following as described in this Technical Manual. The bulk suspension of reporter cells was supplemented with a 2x-EC₇₅ concentration of 17 β -Estradiol and dispensed into the assay plate at 100 μ l/well. Cells were then further treated by adding 100 μ l/well of decreasing 2x-concentrations of antagonist. Assay plates were incubated for ~23 hrs, then processed to quantify ER β activity for each treatment condition.

II. Product Components & Storage Conditions

This Human ER β 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 reporter 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.

<i>Kit Components</i>	<i>Amount</i>	<i>Storage Temp.</i>
▪ ER β 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
▪ 17 β -Estradiol, 600 nM (in DMSO) (E2, reference agonist for ERs)	1 x 30 μ L	-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% CO₂ 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*: antagonist reference compound.
- *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.

▪ A word about Antagonist-mode assay setup ▪

When setting up receptor inhibition assays the Reporter Cells are co-treated with a fixed sub-maximal concentration (typically between EC_{50} – EC_{85}) of the reference agonist AND varying concentrations of the test compound(s). This ER β Assay kit includes a 600 nM stock solution of **17 β -Estradiol (E2)** a potent physiological agonist of ER β that may be used to setup antagonist-mode assays. 30 pM 17 β -Estradiol approximates EC_{80} in this assay. Hence, it is a suitable assay concentration of agonist to be used when screening test compounds for inhibitory activity.

Adding a 2x- EC_{80} concentration of challenge agonist (E2) 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 μ l of treatment media is combined with 100 μ l of pre-dispensed [Reporter Cells + agonist]. Consequently, one must prepare the bulk suspension of Reporter Cells to contain a 2x- EC_{80} concentration of E2 (approximately 24 pM). **APPENDIX 1** provides a dilution scheme that may be used as a guide when preparing cell suspension supplemented with a 2x-E2.

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 consider *Note 5.4*): Prepare Test Compound treatment media for *Agonist-* or *Antagonist-mode* screens.

Total DMSO carried over into assay reactions should not exceed 0.4%.

Note that, in *Step 6*, 100 μ l of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 μ l 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 ER β Assay kit includes a 600 nM stock solution of **17 β -Estradiol**, a potent physiological agonist of ER β that may be used as a reference compound. The following 7-point treatment series, with concentrations presented in 3-fold decrements, provides a complete dose-response: 600, 200, 66.7, 22.2, 7.41, 2.47, and 0.823 pM. Always include 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 surface with a 70% ethanol swab.

Second, retrieve **Reporter Cells** from -80°C storage and immerse in dry ice to transport the tube to a laminar flow hood. When ready, 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 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.

5.) a. Agonist-mode assays. Gently invert the tube of cells several times to disperse cell aggregates and gain a homogenous cell suspension. Without delay, dispense 100 µl of cell suspension into each well of the assay plate.

~ or ~

b. Antagonist-mode assays. Gently invert the tube of cells several times to disperse any cell aggregates, and to gain a homogenous cell suspension. Supplement the bulk suspension of Reporter Cells with the desired 2x-concentration of E2 (refer to "A word about antagonist-mode assay setup", pg. 8). 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.

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-channel 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% CO₂) 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 on 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** and **Detection Buffer** from the refrigerator and place them in a low-light area so that they 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. Program the instrument to perform a single 5 second "plate shake" prior to reading the first assay well. Set read-time to 0.5 second (500 mSec) per well, *or less*.

11.) Immediately before proceeding to *Step 15*, prepare **Luciferase Detection Reagent (LDR)**. Combine 'Detection Buffer' and 'Detection Substrate' by pouring-over their entire volumes into a media basin; rock the basin gently to mix the reagent. The resulting volume of LDR is 12 ml.

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. Cells will remain tightly adhered to well bottoms.

13.) Dispense 100 µl of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for at least 5 minutes. Do not shake the assay plate during this period.

14.) Quantify luminescence.

V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
ERβ Family of Assay Products	
IB00411-32	Human ER β Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB00411-48	Human ER β Reporter Assay System 2x 48 assays in 8-well strips (96-well plate format)
IB00411	Human ER β Reporter Assay System 1x 96-well format assay
IB00412	Human ER β 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.	
Panel of ER Assays	
IB00421-48P	Human ER α and ER β Reporter Assay PANEL 48 assays each in 8-well strips (96-well plate format)

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.

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VI. Limited Use Disclosures

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APPENDIX 1

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

