Zebrafish Estrogen Receptor Alpha
(ERα; ESR1; nr3a1)
Reporter Assay System

3x 32 Assays in 96-well Format
Product # Z00401-32

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Technical Manual
(version 7.1bi)

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INDIGO BIOSCIENCES

Nuclear Receptor & *In Vitro* Toxicology Solutions™

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

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 **Zebrafish (*Danio rerio*) Estrogen Receptor 1** (**nr3a1**), a ligand-dependent transcription factor commonly referred to herein as **zfERα**.

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 zfERα activity. Luciferase gene expression occurs after ligand-bound zfERα 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 reporter 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.*

The principal application of this assay is in the screening of test samples to quantify any functional bioactivity that they may exert against zebrafish ERα. In particular, zebrafish reporter assays are frequently used in the monitoring of environmental samples for the presence of biohazardous chemical pollutants, such as endocrine disruptors.

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's reporter assays are all-inclusive assay systems. In addition to zfERα Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, the reference agonist 17β-estradiol, Luciferase Detection Reagent, and a cell culture-ready assay plate comprised of twelve 8-well strips and a plate frame.

▪ The Assay Chemistry ▪

INDIGO's 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 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 reporter 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.

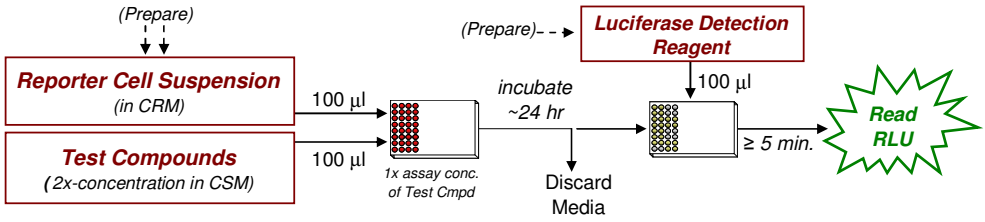
> *NOTE:* The final concentration of total DMSO carried over into assay reactions should not exceed 0.4%.

Immediately prior to setting up the assay plate(s) master stocks are serially diluted using **Compound Screening Medium** (**CSM**; as described in *Step 2 of the Assay Protocol*) to generate *2x-concentrated* treatment media.

> *NOTE:* CSM is formulated to help stabilize hydrophobic test compounds in the aqueous environment of the assay mixture. Nonetheless, high concentrations of hydrophobic test compounds diluted in CSM will lack long-term stability and/or solubility, especially if further stored at low temperatures. Hence, it is advised 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 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.



▪ Assay Performance ▪

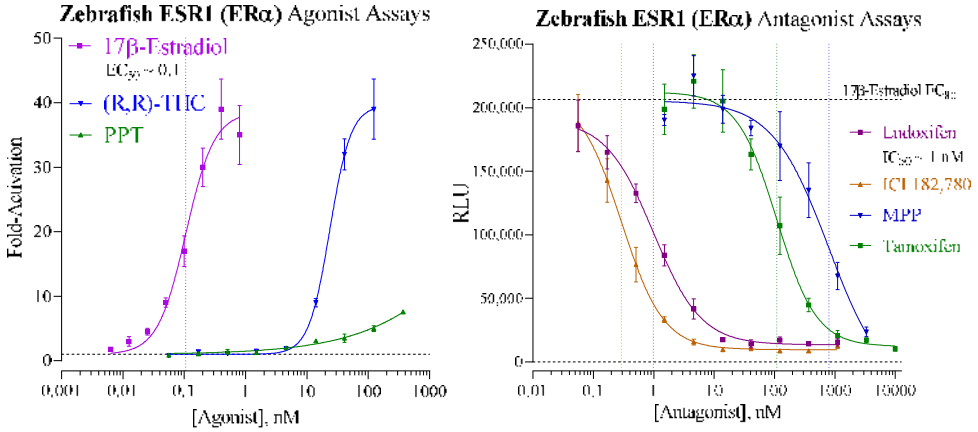


Figure 2. Agonist and Antagonist dose-response analyses of Zebrafish ERα.

zfERα Reporter Cells were treated with the agonists 17β-Estradiol (provided) and (R,R)-THC and PPT. For antagonist assays reporter cells were co-treated with a fixed (EC_{80}) concentration of 17β-estradiol and varying concentrations of the antagonists Endoxifen, ICI 182780, MPP and Tamoxifen citrate (all from Tocris). Assay workflow was as described in this Technical Manual. Luminescence/well was quantified and the average relative light units (RLU), corresponding standard deviation (SD), percent coefficient of variation (%CV) and fold-activation values were determined for each treatment concentration (n = 4). Average fold-activation +/- %CV (agonist assays) or RLU +/- SD (antagonist assays) were plotted against their respective treatment concentrations, Log10 (nM), using GraphPad Prism software.

II. Product Components & Storage Conditions

This Zebrafish ERα assay kit 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.

Kit Components	*Amount*	*Storage Temp.*
▪ zfERα Reporter Cells	3 x 0.6 mL	**-80°C**
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ 17β-Estradiol, 600 nM (in DMSO) (reference agonist for ER's)	1 x 30 µL	-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	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

▪ container of dry ice (used in *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* deep-well plates, *or* appropriate similar vessel for generating dilution series of reference compound(s) and test compound(s).
▪ *Optional:* antagonist reference compound (refer to Fig 2)
▪ *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 ▪

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 assay kit includes a 600 nM stock solution of **17β-Estradiol**, a potent agonist of sf ERα that may be used to setup antagonist-mode assays. 150 pM 17β-Estradiol typically approximates $EC_{70\text{-}80}$ in this assay. Hence, this is a suitable final concentration of agonist to be used when screening test compounds for inhibitory activity.

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, 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 the cell suspension supplemented with the desired 2x-concentration of agonist.

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 the option described in *Note 5.1*): 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 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 assay kit includes a 600 nM stock solution of **17β-Estradiol**, a potent reference agonist of zfERα. The following 8-point treatment series, with concentrations presented in 2-fold decrements, provides a complete dose-response: 600, 300, 150, 75.0, 37.5, 18.8, 9.38 and 4.69 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, sanitize the outside with a 70% ethanol swab, then place it in the cell-culture hood.

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, 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.

5.) ***a. Agonist*-mode assays**. *Gently* invert the tube of Reporter 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 Reporter 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 reference agonist (refer to "*A word about antagonist-mode assay setup*", pg. 8). Then dispense 100 µl / well of the supplemented cell suspension into the assay plate.

NOTE 5.1: For logistical reasons, some users find it more convenient to plate the reporter cells first, and then begin preparing 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 5.2: If INDIGO's Live Cell Multiplex Assay is to be incorporated, a minimum of 3 'blank' wells (meaning cell-free, but containing 'CSM') must be included in the assay plate to allow quantification of fluorescence background (refer to the LCMA Technical Manual).

NOTE 5.3: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling in the reservoir during the dispensing period. Likewise, take care to ensure precision in dispensing exact volumes across the assay plate.

NOTE 5.4: 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.

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

7.) Place the assay plate in a 37°C, humidified 5% CO_2 incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 85%) 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** 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. 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 an 8-pin manifold (*e.g.,* Wheaton Science Microtest Syringe Manifold, # 851381) affixed to a vacuum-trap apparatus.

13.) Add 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

Product No.	*Product Descriptions*
Human ERα Assays	
IB00401-32	Human ERα Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
IB00401	Human ERα Reporter Assay System 1x 96-well format assay
IB00402	Human ERα Reporter Assay System 1x 384-well format assays
Panel of Human ERα / ERβ Assays	
IB00421-48P	Human ERα and ERβ Reporter Assay PANEL 48 assays each, in 8-well strips (96-well plate format)
Rat ERα Assays	
R00401-32	Rat ERα Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
R00401	Rat ERα Reporter Assay System 1x 96-well format assay
Zebrafish ERα Assays	
Z00401-32	Zebrafish ERaa Reporter Assay System 3x 32 assays in 8-well strips (96-well plate format)
Z00401	Zebrafish ERaa Reporter Assay System 1x 96-well format assay
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.

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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, claims and technical protocols are subject to change without prior notice. The printed Technical Manual provided in the kit box will always be the most currently updated version.

APPENDIX 1

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