Human Estrogen-Related Receptor, Alpha

(NR3B1, ERRα)

Reporter Assay System

3x 32 Assays in 96-well Format

Product # IB08001-32

▪

Technical Manual

(version 6.0)

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Human ERRα 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 human mammalian cells engineered to provide high-level expression of a hybrid form of the **Human Estrogen-Related Receptor Alpha (NR3B1)**. The N-terminal DNA binding domains (DBD) of the native ERRα 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 ERRα in a constant state of high-level activity. **Figure 2** demonstrates the constitutive activity of ERRα in the absence of treatment compounds. Therefore, the principle application of this assay system is in the screening of test samples to quantify *inverse-agonist* activities that they may exert against human ERRα.

ERRα 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 ERRα Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, a reference inverse-agonist, reagents to prepare Luciferase Detection Reagent, and a cell culture-ready assay plate.

▪ 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 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 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.

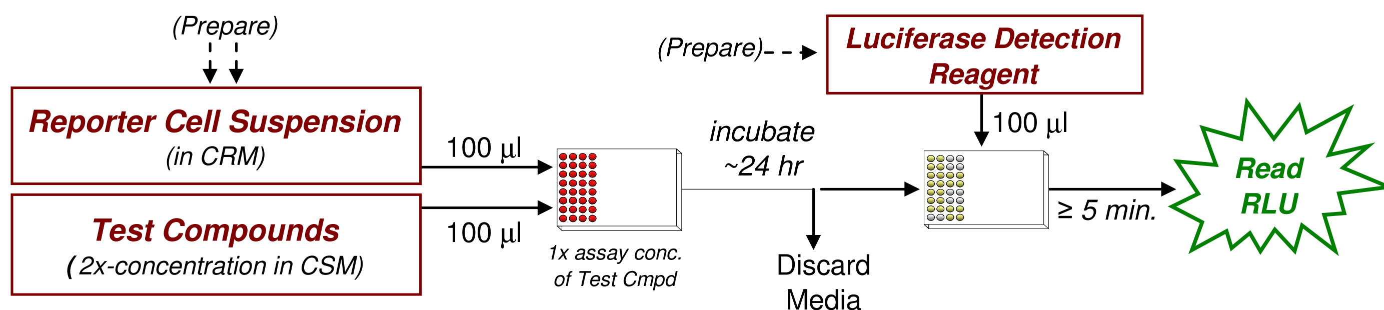
▪ Preparation of Test Compounds ▪

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.



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Human ERRα (NR3B1): Inverse-agonist response to XCT790

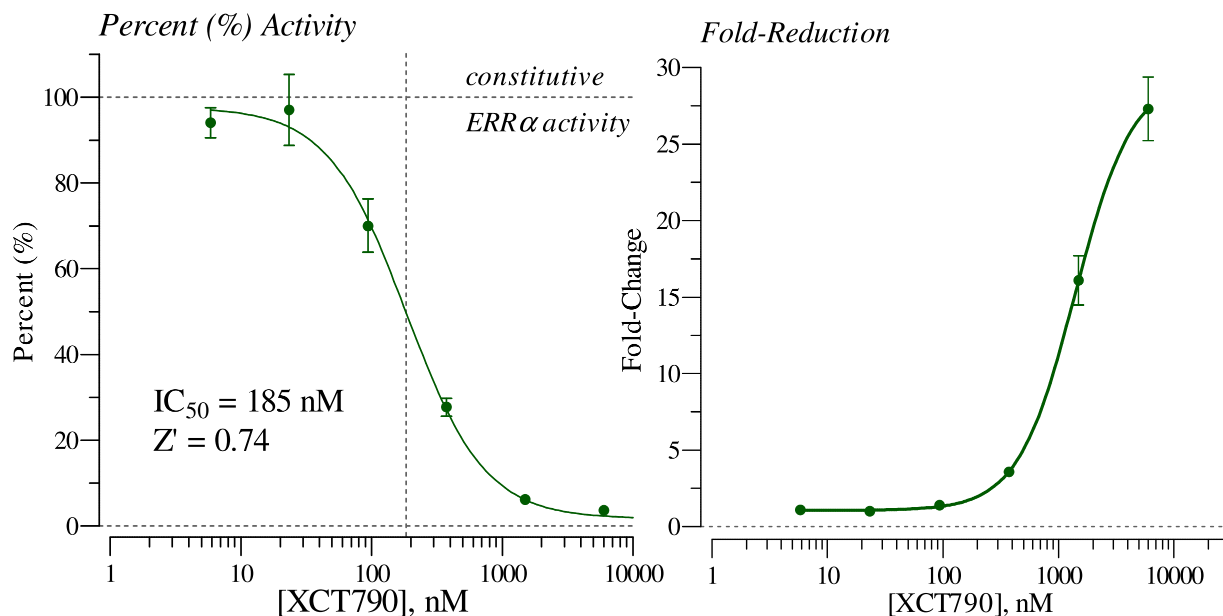

Figure 2. Inverse-agonist dose-response analyses of Human ERRα.

Human ERRα Reporter Cells were treated with varying concentrations of the inverse-agonist XCT790[1] (provided). 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 ($n \geq 6$). Percent change in ERRα activity was 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)[2]. Non-linear regression and IC_{50} determination were performed using GraphPad Prism software.

[1] Bush BB, Stevens WC, Martin R, Ordenthlich P, Zhou S, Sapp DW, Horlick RA and Mohan R. (2004) Identification of a selective inverse agonist for orphan nuclear receptor estrogen-related receptor alpha. J Med Chem.: **47**, 5593-5596.

[2] 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.

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

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II. Product Components & Storage Conditions

This Human ERRα 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.

Kit Components	*Amount*	*Storage Temp.*
▪ ERRα 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
▪ XCT790, 12 mM (in DMSO) (reference inverse-agonist for ERRα)	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	-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

- ▪ 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).

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 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.)** Use **CSM** to prepare appropriate dilution series of Test Compound(s) and Reference Compound stocks to be screened for inverse-agonist 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). Plan dilution schemes carefully; this assay kit provides 35 ml of CSM.

This ERRα Reporter Assay System includes a 12 mM stock solution of all XCT790, an inverse-agonist of ERRα. We find the following 7-point treatment series, prepared in serial 4-fold decrements, provides a suitable dose-response: 24000, 6000, 1500, 375, 93.8, 23.4, and 5.86 nM, 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.

☐ **5.)** 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.

> *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 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 (as described in *Step 2*) into appropriate wells of the assay plate.

☐ **7.)** Transfer the assay plate into a 37°C, humidified 5% CO_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.

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 ERRα 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. 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.

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V. Related Products

Human ERRα Assay Products	
Product No.	***Product Descriptions***
IB08001-32	Human ERRα Reporter Assay System 3x 32 assays in 96-well format
IB08001	Human ERRα Reporter Assay System 1x 96-well format assay
IB08002	Human ERRα 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 Products	
Product No.	***Product Descriptions***
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 in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats
LCM-10	Reagent in 10x-bulk volume to perform **960** Live Cell Assays in any combination of 1x96-, 2x48-, or 3x32-well assay plate formats

Please refer to INDIGO Biosciences website for updated product offerings.

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VI. Limited Use Disclosures

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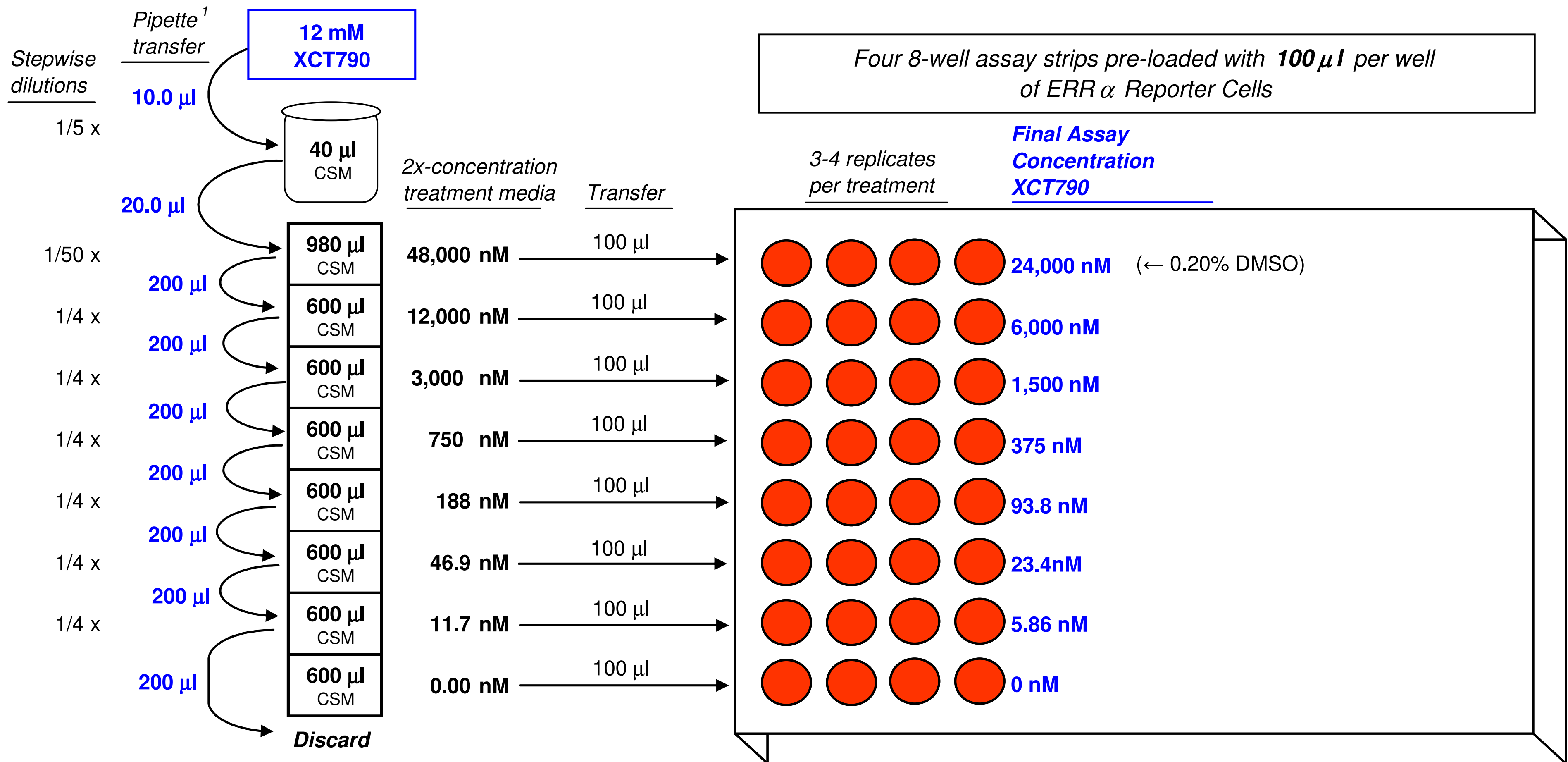
APPENDIX 1

Example scheme for the serial dilution of the inverse-agonist XCT790, and the setup of an ERRα dose-response assay.



[1] For convenience, serial dilutions may be made directly in a multi-well basin or a deep 96-well plate.