

**Human Estrogen-Related Receptor, Alpha
(NR3B1, ERR α)
Reporter Assay System**

96-well Format Assays
Product # IB08001

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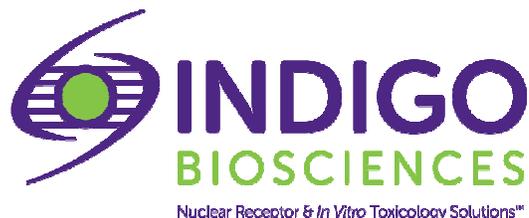
Technical Manual
(version 7.2)

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Human ERR α Reporter Assay System 96-well Format Assays

I. Description	
▪ The Assay System.....	3
▪ The Assay Chemistry.....	3
▪ Preparation of Test Compounds.....	4
▪ Considerations for Automated Dispensing.....	4
▪ Assay Scheme.....	4
▪ Assay Performance.....	5
II. Product Components & Storage Conditions	6
III. Materials to be Supplied by the User	6
IV. Assay Protocol	
▪ <i>DAY 1 Assay Protocol</i>	7
▪ <i>DAY 2 Assay Protocol</i>	9
V. Related Products	9
VI. Limited Use Disclosures	9
APPENDIX 1: Example Scheme for Serial Dilutions	10

I. Description

▪ The Assay System ▪

This nuclear receptor assay 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 ERR α 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 α in a constant state of high-level activity. **Figure 2** demonstrates the constitutive activity of ERR α in the absence of treatment compounds. Therefore, the principal application of this assay is in the screening of test samples to quantify *inverse-agonist* activities that they may exert against human ERR α .

ERR α 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 or cell titer adjustments prior to assay setup.

INDIGO's Nuclear Receptor assays are all-inclusive cell-based assay systems. In addition to ERR α 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 assay kits 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, PPi, 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 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** ▪

Test compounds are typically solvated at high-concentration in DMSO and stored frozen as master stocks. Immediately prior to setting up an assay, the master stocks are serially diluted using **Compound Screening Medium (CSM)**; as described in *Step 7* to achieve the desired assay concentrations. Do not use DMSO to further dilute test compound solutions. This method of dilution avoids the significant 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

▪ **Considerations for Automated Dispensing** ▪

When processing a small number of assay plates, first carefully consider 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.

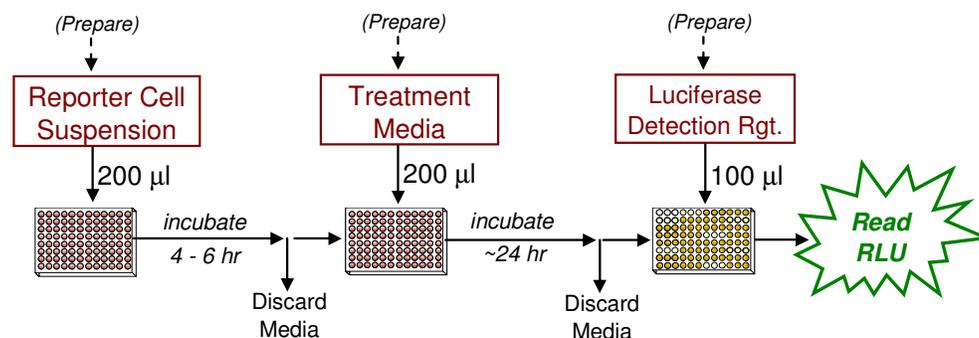
Stock Reagent & Volume provided	Volume to be Dispensed (96-well plate)	Excess rgt. volume available for instrument dead volume
Reporter Cell Suspension 21 ml <i>(prepared from kit components)</i>	200 µl / well 19.2 ml / plate	~ 1.8 ml
LDR 12 ml <i>(prepared from kit components)</i>	100 µl / well 9.6 ml / plate	~ 2.4 ml

▪ **Assay Scheme** ▪

Figure 1. Assay workflow.

NOTE: This ERRα assay protocol includes Day 1 steps and dispensed volumes that differ from the historical protocol that some users may be accustomed to; please review the assay workflow, below.

In brief, 200 µl of Reporter Cells is dispensed into wells of the assay plate and pre-incubated for 4-6 hours. Following the pre-incubation period, culture media are discarded and 200 µl/well of the prepared 1x-concentration treatment media are added. Following 22-24 hr incubation, treatment media are discarded and Luciferase Detection Reagent 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 ▪

Human ERR α (NR3B1): Inverse-agonist response to XCT790

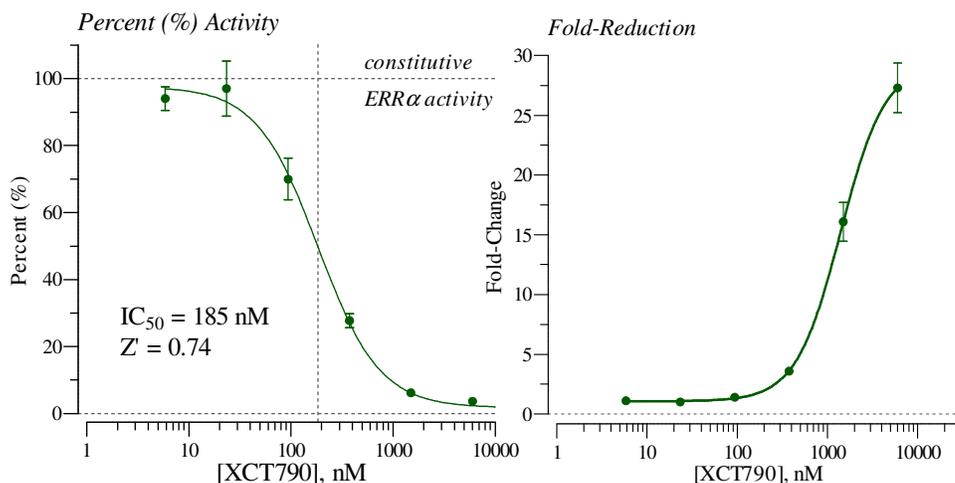


Figure 2. Inverse-agonist dose-response analyses of Human ERR α .

Human ERR α Reporter Cells were treated with varying concentrations of the inverse-agonist XCT790¹ (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 ERR α 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 IC₅₀ determination were performed using GraphPad Prism software.

¹ Bush BB, Stevens WC, Martin R, Ordentlich 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.

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

II. Product Components & Storage Conditions

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

The aliquot of Reporter Cells is provided as a single-use reagent. 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ ERR α Reporter Cells	1 x 2.0 mL	-80°C
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ XCT790, 12 mM (in DMSO) (reference inverse-agonist for ERR α)	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, collagen-coated)	1	-20°C

NOTE: This assay kit contains one 96-well assay plate in which the assay wells have been collagen-coated and dried; the assay plate should be stored frozen (-20°C or colder) until use.

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% CO₂ 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).
- *Optional:* antagonist reference compound.
- *Optional:* clear 96-well assay plate, 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 1-11* are performed on **Day 1**, requiring less than 2 hours of bench work and a 4 hr incubation step to complete. *Steps 12-17* 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 the **2 tubes** of **Cell Recovery Medium (CRM)** from freezer storage, thaw and equilibrate to 37°C using a water bath.

2.) **Rapid Thaw of the Reporter Cells:** *First*, retrieve the two tubes of **CRM** from the 37°C water bath and sanitize their outside surfaces with a 70% ethanol swab.

Second, retrieve the tube of **Reporter Cells** from -80°C storage and, *without delay*, perform a rapid thaw of the frozen cells by transferring 9.5 ml from *each of the 2 tubes* of 37°C CRM into the tube of frozen cells. Place the tube of Reporter Cells in a 37°C water bath for 5 - 10 minutes. The resulting volume of cell suspension will be **21 ml**.

3.) Retrieve the tube of Reporter Cell Suspension from the water bath and sanitize the outside surface with a 70% alcohol swab.

4.) Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain a homogenous cell suspension. Transfer the cell suspension into a reservoir. Using an 8-channel pipette, dispense **200 µl / well** of cell suspension into the assay plate.

NOTE 4.1: Increased well-to-well variation (= increased standard deviation!) will occur if care is not taken to prevent cells from settling during the dispensing period. Likewise, take care to dispense uniform volumes across the assay plate.

NOTE 4.2: 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 into a clear 96-well cell culture treated assay plate. Continue to process the assay plate in identical manner to the white assay plate.

5.) **Pre-incubate reporter cells:** Place the assay plate into a 37°C, ≥ 85% humidity, 5% CO₂ incubator for 4 - 6 hours.

Near the end of the 4-6 hour pre-incubation period:

6.) Remove **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.

7.) **a. Prepare the Test Compound and Reference Compound treatment media at the desired final assay concentrations:** Use CSM to prepare an appropriate dilution series of the reference and test compound stocks. Prepare all treatment media at the desired final assay concentrations. In *Step 9*, the prepared treatment media will be dispensed at 200 µl / well into the assay plate. Manage dilution volumes carefully; this assay kit provides **45 ml** of CSM.

NOTE: Total DMSO carried over into assay reactions should never exceed 0.4%.

b. Preparing the positive control: This ERR α assay kit includes a 12 mM stock solution of all XCT790, an inverse-agonist of ERR α . The following 7-point treatment series, prepared in serial 3-fold decrements, provides a suitable dose-response: 6000, 2000, 667, 222, 74.1, 24.7 and 8.23 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

8.) At the end of the cell pre-incubation period, **discard the culture media** by 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.

- 9.) Dispense **200 μ l** of each treatment media into appropriate wells of the assay plate.
- 10.) Transfer the assay plate into a 37°C, humidified 5% CO₂ incubator for 22 - 24 hours.
NOTE: Ensure a high-humidity (\geq 85%) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.
- 11.) 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.

- 12.) 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. Once at 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.
- 13.) 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*.
- 14.) *Immediately before proceeding to Step 15*, 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.
- 15.) Following 22 - 24 hours incubation in treatment media, discard the media contents by 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.
- 16.) Add 100 μ l of **LDR** to each well of the assay plate. 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.
- 17.) Quantify luminescence.

V. Related Products

Human ERRα Assay Products	
<i>Product No.</i>	<i>Product Descriptions</i>
IB08001-32	Human ERR α Reporter Assay System 3x 32 assays in 96-well format
IB08001	Human ERR α Reporter Assay System 1x 96-well format assay
IB08002	Human ERR α 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	
<i>Product No.</i>	<i>Product Descriptions</i>
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

Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans.

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APPENDIX 1

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

