Human Estrogen-Related Receptor, Alpha
(NR3B1, ERRα)
Reporter Assay System

3x 32 Assays in 96-well Format
Product # IB08001-32

Technical Manual
(version 7.2)

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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 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 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 or cell titer adjustments prior to assay setup.

INDIGO’s Nuclear Receptor 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 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 Mg²⁺-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 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%.

▪ 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 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 ≥ 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.


\[ Z' = 1 - \left( \frac{3 \times (SD_{\text{Control}} + SD_{\text{Background}})}{(RLU_{\text{Control}} - RLU_{\text{Background}})} \right) \]
II. Product Components & Storage Conditions

This Human ERRα 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.

The 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.

<table>
<thead>
<tr>
<th>Kit Components</th>
<th>Amount</th>
<th>Storage Temp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>• ERRα Reporter Cells</td>
<td>3 x 0.6 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Cell Recovery Medium (CRM)</td>
<td>2 x 10.5 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Compound Screening Medium (CSM)</td>
<td>1 x 45 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• XCT790, 12 mM (in DMSO) (reference inverse-agonist for ERRα)</td>
<td>1 x 30 µL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Detection Substrate</td>
<td>3 x 2.0 mL</td>
<td>-80°C</td>
</tr>
<tr>
<td>• Detection Buffer</td>
<td>3 x 2.0 mL</td>
<td>-20°C</td>
</tr>
<tr>
<td>• Plate frame</td>
<td>1</td>
<td>ambient</td>
</tr>
<tr>
<td>• Snap-in, 8-well strips (white, sterile, collagen-coated wells)</td>
<td>12</td>
<td>-80°C</td>
</tr>
</tbody>
</table>

NOTE: This Assay kit contains 8-well strips that have been collagen-coated and dried; these strip wells 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 Reporter Cells from -80°C storage: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. *Without delay*, perform a rapid thaw of the frozen cells by transferring 6.4 ml of pre-warmed CRM into each 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 7.0 ml per tube.

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

4.) If more than one tube of Reporter cells was thawed, combine them and gently invert several times to disperse cell aggregates and gain a homogenous cell suspension. 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 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. Prepare the positive control:* This ERRα assay kit includes a 12 mM stock solution of all XCT790, an inverse-agonist of ERRα. 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. Because the assay plate is composed of a frame with snap-in strip-wells, the practice of physically ejecting media 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. 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 cells and greatly increased well-to-well variability.

9.) Dispense 200 µl 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 (≥ 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 the appropriate number of vials of Detection Substrate and Detection Buffer from freezer storage and place them in a dark refrigerator (4°C) to thaw overnight.

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:* 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.

15.) Following 22 - 24 hours incubation in treatment media, remove media contents from each well of the assay plate (as before in Step 8).

16.) 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 following the addition of LDR. Do not shake the assay plate during this period.

17.) Quantify luminescence.
V. Related Products

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
</table>
| 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.

<table>
<thead>
<tr>
<th>Product No.</th>
<th>Product Descriptions</th>
</tr>
</thead>
</table>
| 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.

[www.indigobiosciences.com](http://www.indigobiosciences.com)

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.