

**Human RAR-related Orphan Receptor, Alpha  
(NR1F1, RORA, ROR $\alpha$ )  
Reporter Assay System**

**96-well Format Assays**  
Product # IB04011

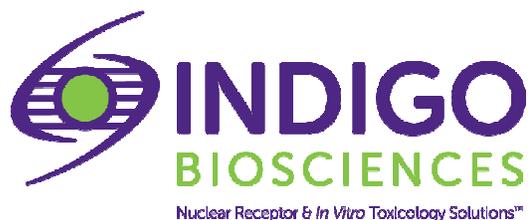
*(version 7.3b)*

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## Human ROR $\alpha$ Reporter Assay System 96-well Format Assays

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## I. Description

### ▪ The Assay System ▪

This nuclear receptor assay utilizes proprietary non-human mammalian cells engineered to provide high-level expression of a hybrid form of the **Human RAR-related Orphan Receptor Alpha (NR1F1)**. The N-terminal DNA binding domains (DBD) of the native ROR $\alpha$  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 ROR $\alpha$  in a constant state of high-level activity. **Figure 2** demonstrates the constitutive activity of ROR $\alpha$  in the absence of added ligand, and the dose-dependent *loss* of constitutive activity upon treatment with compounds exerting inverse-agonist activity. Conversely, **Figure 3** demonstrates that ligand interactions can also result in agonist responses, producing moderate increases in ROR $\alpha$  activity above its already high level of constitutive activity. Therefore, the principal applications of this assay are in the screening of test samples to quantify *inverse-agonist* and/or *agonist* activities that they may exert against human ROR $\alpha$ .

INDIGO's Nuclear Receptor Assay kits are all-inclusive cell-based assay systems. In addition to ROR $\alpha$  Reporter Cells, this kit provides two optimized media for use during cell culture and in diluting the user's test samples, a buffer for rinsing assay wells, the reference inverse-agonist ATRA, 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<sup>+2</sup>-dependent reaction that consumes O<sub>2</sub> and ATP as co-substrates, and yields as products oxyluciferin, AMP, PP<sub>i</sub>, CO<sub>2</sub>, 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 ▪

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 inverse-agonist ATRA, **Compound Screening Medium (CSM)** may be used as the diluent to make serial dilutions of test compounds to achieve the desired final 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 final concentration of total DMSO carried over into assay wells should *never* 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 that they are considered to be 'single-use' reagents.

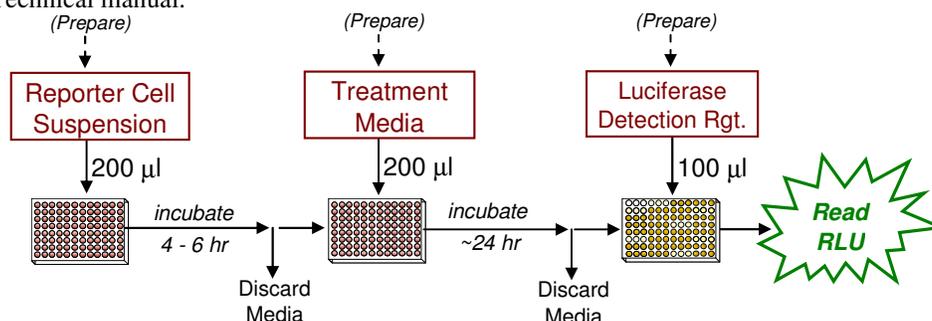
### ▪ Considerations for Automated Dispensing ▪

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.

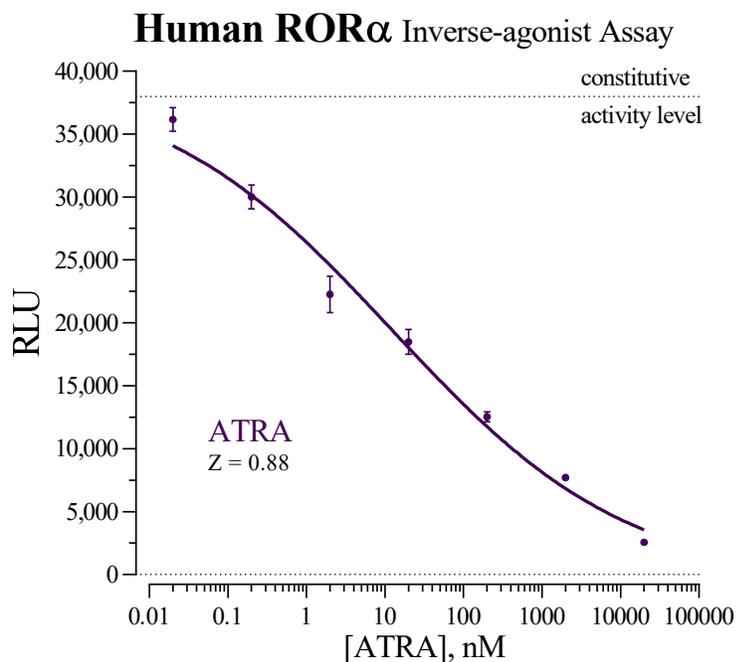
Stock Reagent & Volume provided	Volume to be Dispensed (96-well plate)	Excess rgt. volume available for instrument dead volume
<b>Reporter Cell Suspension</b> 21 ml (prepared from kit components)	200 µl / well 19.2 ml / plate	~ 1.8 ml
<b>LDR</b> 12 ml (prepared from kit components)	100 µl / well 9.6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

### ▪ Assay Scheme ▪

**Figure 1.** Assay workflow. *In brief*, Reporter Cells 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 the prepared 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. *Note:* If INDIGO's Live Cell Multiplex (LCM) Assay is to be incorporated, refer to the assay workflow schematic provided in the LCM Assay Technical manual.



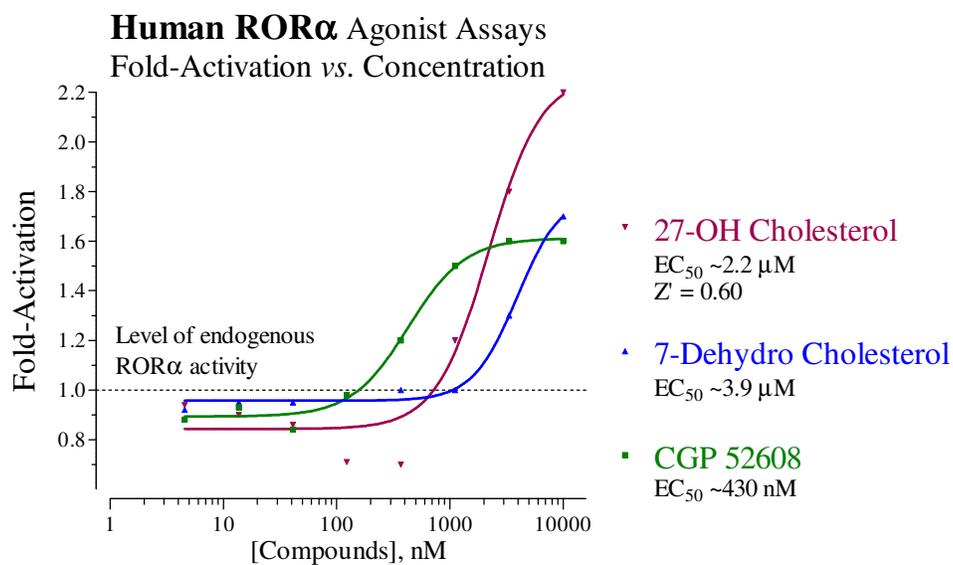
▪ Assay Performance ▪



**Figure 2. Inverse-agonist dose-response performance of the Human ROR $\alpha$  assay.**

Inverse-agonist analyses of Human ROR $\alpha$  Reporter Cells using All *trans*-Retinoic Acid (ATRA; 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 ( $n \geq 6$ ).  $Z'$  value was calculated as described by Zhang, *et al.* (1999)<sup>1</sup>. Non-linear regression was performed using GraphPad Prism software.

<sup>1</sup> 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.



**Figure 3. Agonist dose-response performance of the Human ROR $\alpha$  assay.**

Agonist response of the Human ROR $\alpha$  Reporter Cells is demonstrated using 27-Hydroxy Cholesterol (27OHC; Cayman Chemical), 7-dehydro Cholesterol (Sigma), and CGP 52608 (Sigma).

Values of *Fold-Activation* are plotted against concentration. 27OHC provides greater than a 2-fold increase in ROR $\alpha$  activity above the already high endogenous activity level. Z' values confirm the robust performance of the agonist-mode ROR $\alpha$  assay.

When contemplating concentration ranges for screening test compounds of unknown bioactivity, it is important to note the great disparity in potencies between inverse-agonists and these agonist reference compounds: ATRA IC<sub>50</sub> < 1 nM, whereas 27OHC > 1  $\mu$ M.

## II. Product Components & Storage Conditions

This Human ROR $\alpha$  Assay kit contains materials to perform assays in a single collagen-coated 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 2 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ ROR $\alpha$ Reporter Cells	1 x 2.0 mL	<b>-80°C</b>
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ all <i>trans</i> -Retinoic Acid, 10 mM (in DMSO) (reference inverse-agonist for ROR $\alpha$ )	1 x 30 $\mu$ L	-20°C
▪ Detection Substrate	1 x 6.0 mL	<b>-80°C</b>
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well assay plate (white, sterile, collagen-coated)	1	<b>-20°C</b>

*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

- dry ice bucket (*Step 2*)
- cell culture-rated laminar flow hood.
- 37°C, humidified 5% CO<sub>2</sub> 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:* clear 96-well assay plate, sterile, *collagen-coated*, 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 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, place it directly into a dry ice bucket and transport the cells to the laminar flow hood. When ready, transfer the tube of reporter cells into a rack and, *without delay*, perform a rapid thaw of the 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 gain a homogenous cell suspension, then 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:* 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 4.2:* 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 4.3:* 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 into a clear, *collagen-coated* 96-well assay plate. Continue to process the clear plate in identical manner to the white assay plate.

**5.) Pre-incubate reporter cells.** Place the assay plate into a cell culture incubator (37°C, ≥ 70% humidity, 5% CO<sub>2</sub>) 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.) **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%.

**Prepare the treatment media at the desired final assay concentrations:** This ROR $\alpha$  Assay kit includes a 10 mM stock solution of ATRA, an inverse-agonist of ROR $\alpha$ . The following 8-point treatment series, prepared in serial 10-fold decrements, provides a suitable dose-response range: 20000, 2000, 200, 20, 2.0, 0.20, 0.020 and 0.0020 nM, and including a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

8.) At the end of the 4-6 hr pre-culture period, discard the media. The preferred method is to use a 'wrist flick' to eject media 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 µl / well** of each prepared treatment media into the assay plate.

NOTE: 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

10.) Transfer the assay plate into a cell culture incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity ( $\geq 70\%$ ) 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 an open 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.

*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 all 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 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

Product No.	Product Descriptions
<b>Human ROR<math>\alpha</math> Assay Kit Products</b>	
IB04011-32	Human ROR $\alpha$ Reporter Assay System 3x 32 assays; 8-well strips in 96-well format plate frame
IB04011	Human ROR $\alpha$ Reporter Assay System 1x 96-well format assays
Bulk assay reagents may be custom manufactured to accommodate any scale of HTS. Please Inquire.	

<b>LIVE Cell Multiplex (LCM) Assay Products</b>	
LCM-01	Reagent volumes sufficient to perform <b>96</b> Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagent in <b>5x bulk volume</b> to perform <b>480</b> Live Cell Assays contained in 5 x 96-well assay plates
LCM-10	Reagent in <b>10x bulk volume</b> to perform <b>960</b> Live Cell Assays contained in 10 x 96-well assay plates

Please refer to INDIGO Biosciences website for updated product offerings.

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## VI. Limited Use Disclosures

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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.

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