Human RAR-related Orphan Receptor, Alpha
(NR1F1, RORA, RORα)
Reporter Assay System

96-well Format Assays
Product # IB04011

(version 7.2c)

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INDIGO BIOSCIENCES
The Nuclear Receptor Experts™

Human RORα Reporter Assay System
96-well Format Assays

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I. Description

▪ The Assay System ▪

This nuclear receptor assay system 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 RORα 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 RORα in a constant state of high-level activity. **Figure 2** demonstrates the constitutive activity of RORα 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 RORα activity above its already high level of constitutive activity. Therefore, the principle 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 RORα.

INDIGO Bioscience's Nuclear Receptor Reporter Assays are all-inclusive cell-based assay systems. In addition to RORα 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, 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. Immediately prior to assay setup, concentrated master stocks are diluted using **Compound Screening Medium (CSM)** to generate the desired final assay concentrations, as described in *Step 7* of the **Assay Protocol**. 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.

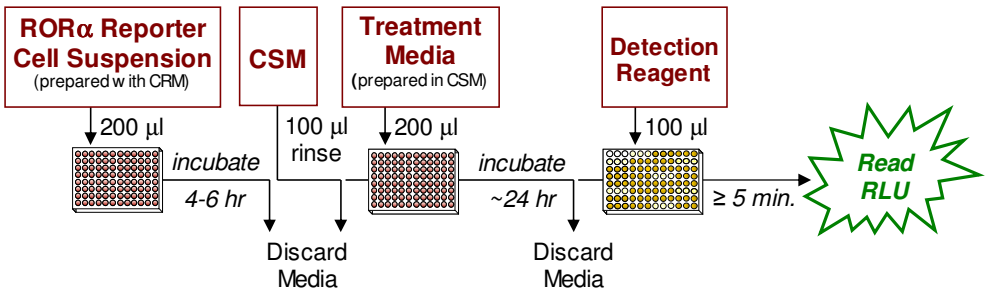
Stock Reagent & Volume provided	**Volume to be Dispensed** *(96-well plate)*	**Excess rgt. volume available for instrument dead volume**
Reporter Cell Suspension 21 ml *(prepared from kit components)*	200 µl / well 19.2 ml / plate	~ 1.8 ml
LDR 12 ml *(prepared from kit components)*	100 µl / well 9.6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

▪ Assay Scheme ▪

Figure 1. Assay workflow.

NOTE: This RORα assay protocol includes steps and dispensed volumes that are different from the conventional INDIGO assay protocol that users may be accustomed to when setting up INDIGO's other Nuclear Receptor Assays.

In brief, 200 µl/well of RORα Reporter Cells are dispensed into the assay plate and pre-incubated for 4-6 hr. Pre-incubation media are removed and wells are briefly rinsed with 100 µl/well of CSM. The rinse media is removed and 200 µl/well of prepared treatment media are added. Following 22 -24 hr incubation, treatment media are discarded and 100 µl/well of prepared Luciferase Detection Reagent (LDR) is added. Light emission (values of relative light units; RLU) from each assay well is quantified using a plate-reading luminometer.



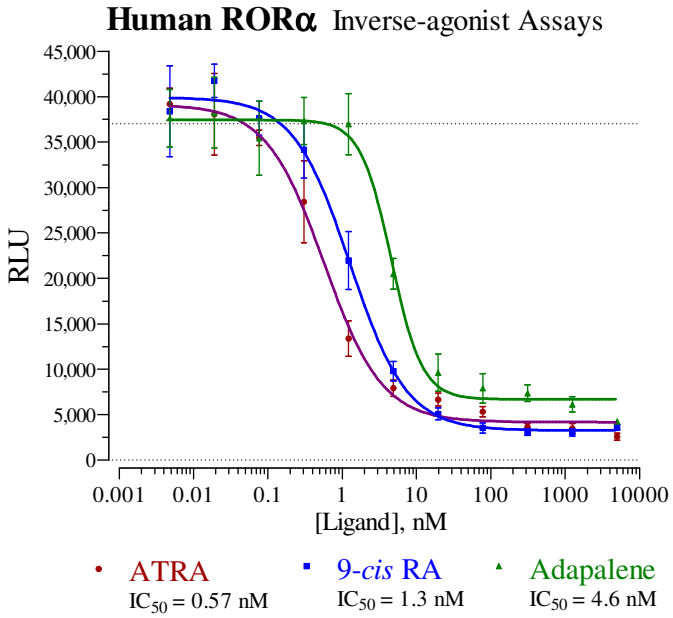


Figure 2. Inverse-agonist dose-response performance of the Human RORα assay.

Inverse-agonist analyses of Human RORα Reporter Cells using All *trans*-Retinoic Acid (ATRA; provided), 9-*cis*-Retinoic Acid (9-*cis*-RA) and Adapalene (both from Cayman Chemical, USA). 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 ($n \geq 6$). Fold-reduction and Z' values were calculated as described by Zhang, *et al*. (1999)[1]. Non-linear regression and IC_{50} analyses were performed using GraphPad Prism software.

[1] 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.

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

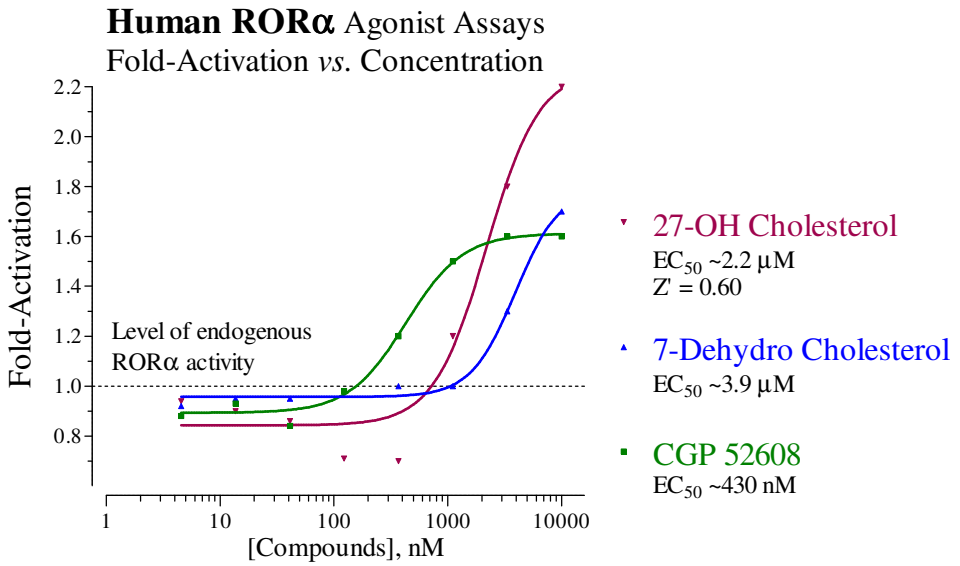


Figure 3. Agonist dose-response performance of the Human RORα assay.

Agonist response of the Human RORα 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 RORα activity above the already high endogenous activity level. Z' values confirm the robust performance of the agonist-mode RORα 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 theses agonist reference compounds: ATRA IC_{50} < 1 nM, whereas 27OHC > 1 µM.

II. Product Components & Storage Conditions

This Human RORα Reporter Assay System contains materials to perform assays in a single 96-well assay plate.

The aliquot of RORα 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.

Kit Components	***Amount***	***Storage Temp.***
▪ RORα 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
▪ all *trans*-Retinoic Acid, 10 mM (in DMSO) (reference inverse-agonist for RORα)	1 x 30 µL	-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% 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 control 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 1-12* are performed on **Day 1**, requiring less than 2 hours to complete. *Steps 13-19* 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 and, *without delay*, perform a rapid thaw of the frozen cells by transferring a 9.5 ml volume from *each* of the 2 tubes of pre-warmed CRM into the 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 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 an homogenous cell suspension. Dispense **200 µl/well** of cell suspension into the 96-well Assay Plate.

NOTE 4.1: 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 4.2: 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 (200 µl/well) into a clear 96-well cell culture treated assay plate. Process the clear assay plate in identical manner to those reporter cells contained in the white assay plate.

5.) Pre-incubate reporter cells: Place the assay plate into a 37°C, ≥ 85% humidity, 5% CO_2 incubator for 4 - 6 hours.

6.) Near the end of the pre-incubation period, remove **Compound Screening Medium (CSM)** from freezer storage and thaw in a 37°C water bath.

7.) Prepare dilutions of test compound treatment media at the desired final assay concentrations: Use **CSM** to prepare appropriate dilution series of test compound stocks. Prepare treatment concentrations at the desired *final* assay concentrations. In *Step 9*, the prepared treatment media are dispensed at **200 µl/well** into the desired number of replicate assay wells. 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 positive control treatment media at the desired final assay concentrations: This RORα Assay kit includes a 10 mM stock solution of ATRA, a potent inverse-agonist of RORα. 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.

At the end of the 4-6 hr cell pre-incubation period:

8.) 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.) Rinse assay wells: Dispense 100 µl of CSM into wells of the assay plate. Briefly manually swirl the plate to rinse the wells, then discard the rinse media.

10.) Dispense 200 µl of prepared treatment media into appropriate wells of the assay plate.

11.) Transfer the assay plate into 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.

12.) 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.

13.) Approximately 30 minutes before intending to quantify RORα 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.

14.) 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. Set "read time" to 0.5 second (500 mSec) per well, *or less*.

15.) *Immediately before proceeding to Step 16*, 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.

16.) Following 22 - 24 hours of incubation, retrieve the assay plate from the incubator. 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.

17.) Add 100 µl of **LDR** to each well of the assay plate.

18.) 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.

19.) Quantify luminescence.

V. Related Products

Human RORα Assay Kit Products	
Product No.	*Product Descriptions*
IB04011-32	3x 32 RORα assays; strip-wells in 96-well plate frame
IB04011	1x 96-well format RORα assays
Bulk 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	Reagents to perform **96** Live Cell Assays in 1x96-well, or 2x48-well, or 3x32-well assay plate formats
LCM-05	Reagents 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

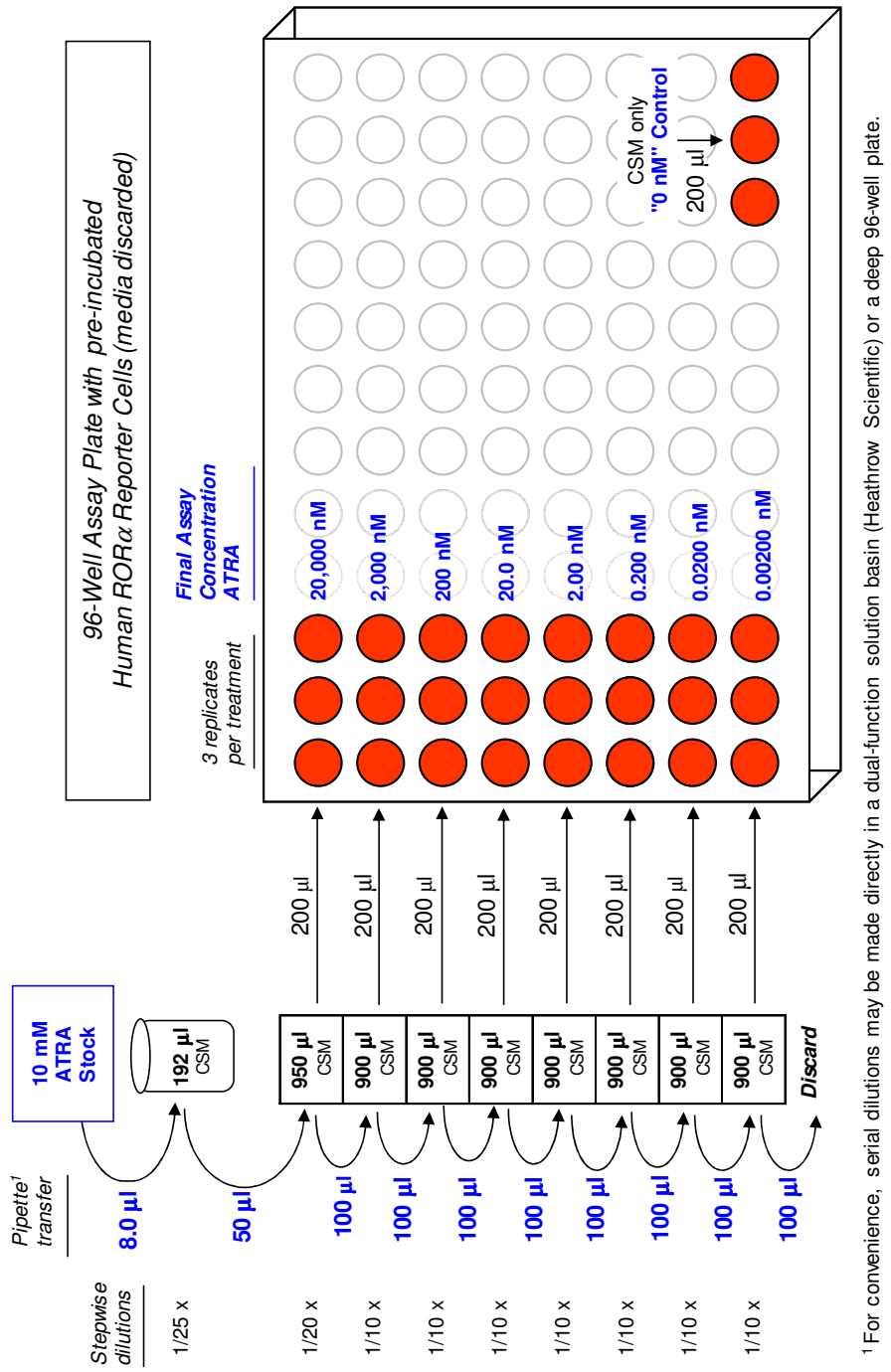
Products commercialized by INDIGO Biosciences, Inc. are for RESEARCH PURPOSES ONLY – not for therapeutic or diagnostic use in humans.

"CryoMite" is a Trademark ™ of INDIGO Biosciences, Inc. (State College, PA)

Product prices, availability, specifications and claims are subject to change without prior notice.

APPENDIX 1

Example scheme for the serial dilution of all *trans*-Retinoic Acid and the setup of an RORα activation dose-response assay.



[1] For convenience, serial dilutions may be made directly in a dual-function solution basin (Heathrow Scientific) or a deep 96-well plate.