

**Human RAR-related Orphan Receptor, Gamma  
(NR1F3, RORC, ROR $\gamma$ )  
Reporter Assay System**

**96-well Format Assays**  
Product # IB04001

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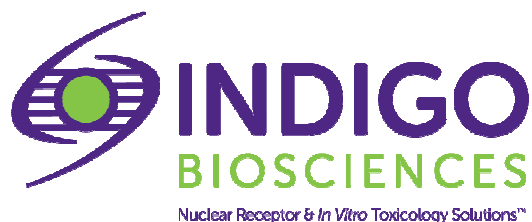
**Technical Manual**  
*(version 7.2im)*

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

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## I. Description

### ▪ The Assay System ▪

The native **Human RAR-related Orphan Receptor Gamma (ROR $\gamma$ )** mRNA is expressed from the RORC gene in two forms *via* alternate usage of tissue-specific promoters. *Variant 1* mRNA is expressed in numerous tissues, and encodes receptor isoform 1, referred to as **ROR $\gamma$** . *Variant 2* mRNA comprises an alternate exon 1 that replaces the exon 1 and 2 sequences found in the Variant 1 transcript. Consequently, variant 2 mRNA presents a unique 5'UTR and modified N-terminal ORF sequences, resulting in the expression of a shorter isoform 2 receptor. The isoform 2 receptor is expressed predominantly in specialized immune cells developing within the thymus; as such it is referred to as **ROR $\gamma$ t**.

This nuclear receptor assay utilizes proprietary human cells engineered to provide high-level expression of a hybrid form of the **Human RAR-related Orphan Receptor Gamma**. The N-terminal DNA binding domains (DBD) of the native ROR $\gamma$  and ROR $\gamma$ t receptors have been substituted with that of the yeast GAL4-DBD. Hence, the GAL4-ROR $\gamma$  hybrid receptor expressed in these reporter cells will not discern any functional differences that may exist between the native isoform 1 and isoform 2 receptors.

As is true *in vivo*, these reporter cells express ROR $\gamma$  in a constant state of high-level activity. **Figure 2** demonstrates the constitutive activity of ROR $\gamma$  in the absence of treatment compounds. Therefore, the principal application of this assay is in the screening of test samples to quantify *inverse-agonist* or *agonist* activities that they may exert against human ROR $\gamma$ .

Reporter Cells are prepared using INDIGO's proprietary **CryoMite™** process. This cryo-preservation method yields high cell viability post-thaw, and provides the convenience of immediately dispensing healthy, division-competent reporter cells into assay plates. There is no need for intermediate spin-and-wash steps or viability determinations prior to plating the reporter cell suspension.

### ▪ The Assay Chemistry ▪

INDIGO's reporter assays 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 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 ML-209, **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 not exceed 0.4%. The emergence of DMSO-induced cytotoxicity can be expected above 0.4% DMSO over a 22-hour treatment period.

*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** ▪

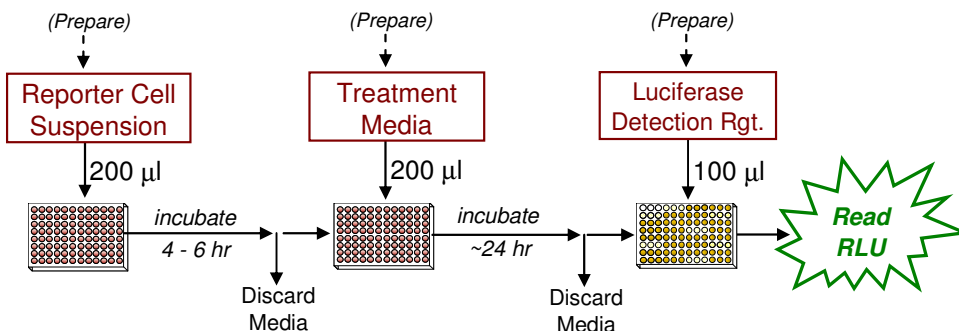
When processing a small number of assay plates, first carefully consider the dead volume requirement of your dispensing instrument before committing assay reagents to its setup. In essence, "dead volume" is the volume of reagent that is dedicated to the instrument; it will *not* be available for final dispensing into assay wells. The following Table provides information on reagent volume requirements, and available excesses.

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

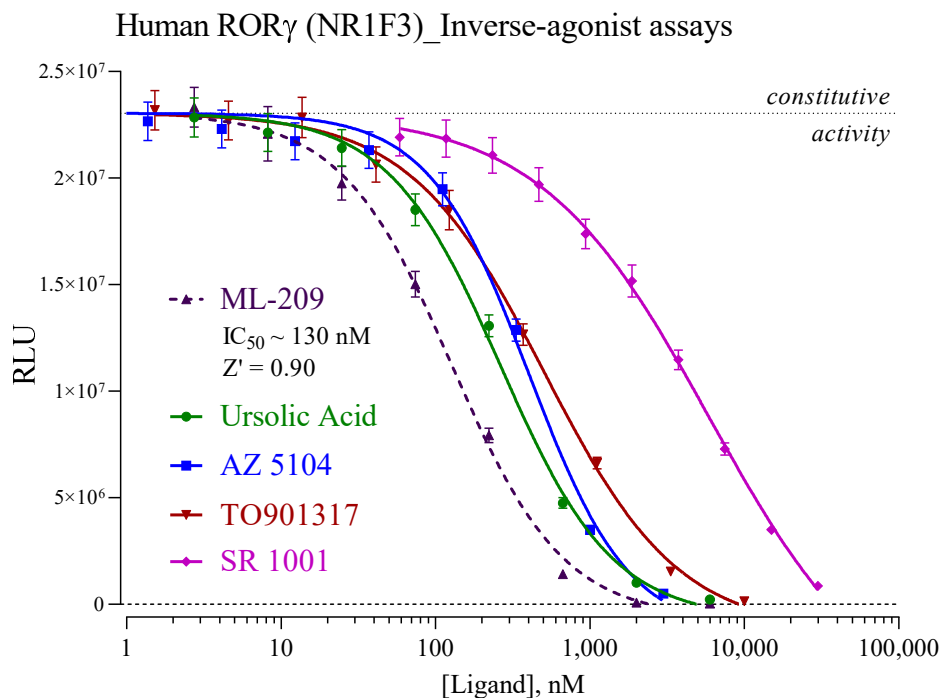
▪ Assay Scheme ▪

**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 an additional 22 - 24 hours 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:* When performing 'loss of activity' assays (*i.e.*, inverse-agonist or antagonist assays) it is advised to always assess the occurrence of compound-induced cytotoxicity. INDIGO's Live Cell Multiplex (LCM) Assay is designed specifically for that purpose. If the LCM Assay is to be incorporated, please refer to the assay workflow schematic provided in the LCM Assay Technical manual.



▪ Assay Performance ▪



**Figure 2. The Human ROR $\gamma$  Assay.** Dose-response analyses of the ROR $\gamma$  Assay were performed using the inverse-agonists ML-209 (provided as reference), Ursolic Acid, AZ 5104, TO901317, and SR 1001 (all from Cayman Chemical, USA). ROR $\gamma$  Assay setup was performed as described in this Technical Manual. Relative Light Units were quantified and average values of RLU and their respective values of relative standard deviation were determined for each treatment concentration. Non-linear regression analyses were performed and IC<sub>50</sub> values determined using GraphPad Prism software. The high Z' value<sup>1</sup> confirms the robust performance of this assay, and its suitability for HTS applications.

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

## II. Product Components & Storage Conditions

This Human ROR $\gamma$  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 $\gamma$ 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
▪ ML-209, 6.0 mM (in DMSO) (reference inverse-agonist for ROR $\gamma$ )	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, <i>collagen-coated</i> assay plate (white, sterile, cell-culture ready)	1	<b>-20°C</b>

*NOTE:* This ROR $\gamma$  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 actual bench work plus a 4-hour pre-incubation step. *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 LCM Assay 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 mammalian cell culture incubator (37°C, ≥70% humidity, 5% CO<sub>2</sub>) 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.) 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  $\mu$ l / 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 not exceed 0.4%.

**b. Prepare the positive control:** This ROR $\gamma$  Assay kit includes a 6.0 mM stock solution of ML-209, a reference inverse-agonist of human ROR $\gamma$ . The following 7-point concentration range, presented in 3-fold decrements, provides a complete inverse-agonist dose-response without cytotoxic effects: 6000, 2000, 667, 222, 74.1, 24.7, and 8.23 nM. Always include a 'no treatment' control. **APPENDIX 1** provides an example for generating such a dilution series.

**8.)** At the end of the 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  $\mu$ l / 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  $\mu$ l 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 therefore may be performed on a bench top.

**12.)** Approximately 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 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.

**16.)** Add 100  $\mu$ l of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for 5 - 10 minutes following the addition of LDR. Do not shake the assay plate during this period.

**17.)** Quantify luminescence.



## V. Related Products

<i>Product No.</i>	<i>Product Descriptions</i>
<b>Human ROR<math>\gamma</math> Assays</b>	
IB04001-32	Human ROR $\gamma$ Reporter Assay System 3x 32 assays; 8-well strips in 96-well format plate frame
IB04001	Human ROR $\gamma$ Reporter Assay System 1x 96-well format assay
IB04002	Human ROR $\gamma$ Reporter Assay System 1x 384-well format assay
<b>Mouse ROR<math>\gamma</math> Assays</b>	
M04001-32	Mouse ROR $\gamma$ Reporter Assay System 3x 32 assays; 8-well strips in 96-well format plate frame
M04001	Mouse ROR $\gamma$ Reporter Assay System 1x 96-well format assay
<b>Rat ROR<math>\gamma</math> Assays</b>	
R04001-32	Rat ROR $\gamma$ Reporter Assay System 3x 32 assays; 8-well strips in 96-well format plate frame
R04001	Rat ROR $\gamma$ Reporter Assay System 1x 96-well format assay
Bulk volumes of 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
<b>INDIGlo Luciferase Detection Reagent</b>	
LDR-10, -25, -50, -500	INDIGlo Luciferase Detection Reagents in 10 mL, 25 mL, 50 mL, and 500 mL volumes

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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# APPENDIX 1

Example scheme for the serial dilution of the inverse-agonist ML-209, and the setup of an ROR $\gamma$  dose-response assay.

