

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

3x 32 Assays in 96-well Format
Product # IB04001-32

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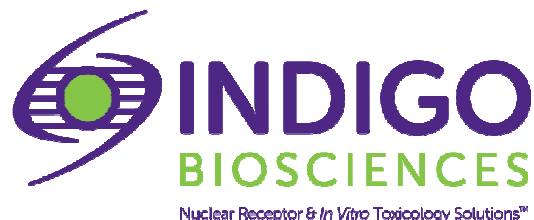
Technical Manual
(version 7.2)

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Human ROR γ Reporter Assay System 3x 32 Assays in 96-well Format

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I. Description

▪ The Assay System ▪

The native **Human RAR-related Orphan Receptor Gamma (ROR γ)** 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 γ** . *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 γ 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 γ and ROR γ t receptors have been substituted with that of the yeast GAL4-DBD. Hence, the GAL4-ROR γ 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 γ in a constant state of high-level activity. **Figure 2** demonstrates the constitutive activity of ROR γ 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 γ .

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 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, 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 ▪

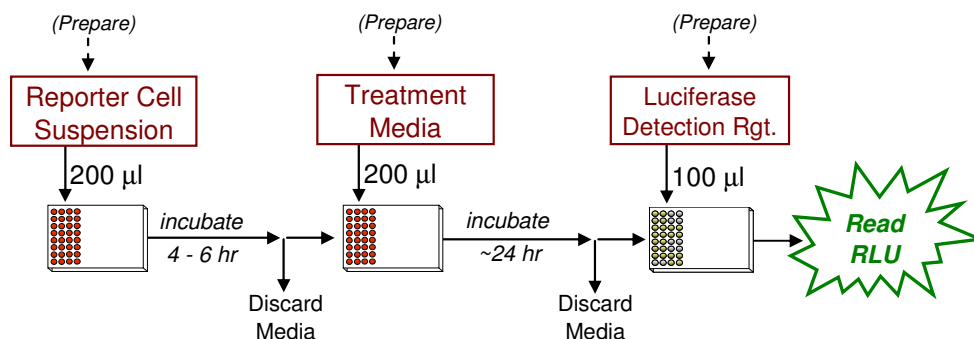
Test compounds are typically solvated at high-concentration in DMSO and stored frozen as master stocks. Immediately prior to setting up an assay, the master stocks are serially diluted using **Compound Screening Medium (CSM)**; as described in *Step 7* to achieve the desired assay concentrations. Do 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 RORγ 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 ▪

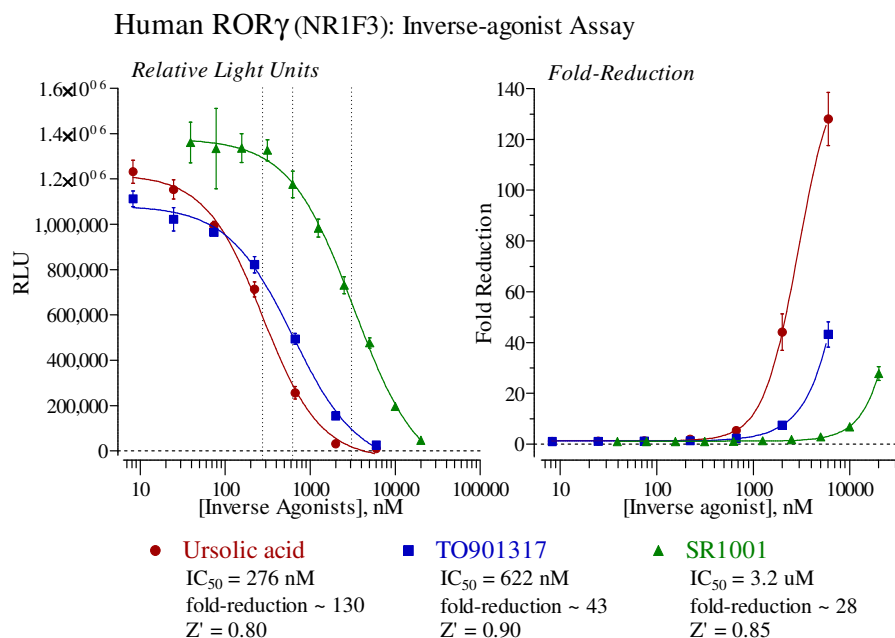


Figure 2. The Human ROR γ Assay.

Dose-response analyses of the ROR γ Assay were performed using the inverse-agonists Ursolic Acid² (provided), TO901317³ (Tocris), and SR1001⁴ (Cayman Chemical). ROR γ Assay setup and quantification were performed as described in this Technical Manual. Relative Light Units (RLU) were quantified using a GloMax-Multi+ plate-reading luminometer (Promega Corp.). Average values of RLU and the respective values of standard deviation (SD) and percent coefficient of variation (%CV) were determined for each treatment concentration. Non-linear regression analyses were performed and IC₅₀ values determined using GraphPad Prism software.

Results: Ursolic acid demonstrates dose-dependent reduction of human ROR γ with an IC₅₀ \leq 280 nM. The large assay window and corresponding high Z' value¹ confirm the robust performance of this assay, and its suitability for HTS applications.

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

²Xu, T., *et al.* (2011) Ursolic acid suppresses interleukin-17 (IL-17) production by selectively antagonizing the function of ROR gamma t protein. *J. Biol. Chem.* 286, 22702-22710.

³Kumar, N., *et al.* (2010) The benzenesulfoamide T0901317 [N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide] is a novel retinoic acid receptor-related orphan receptor-alpha/gamma inverse agonist. *Mol. Pharmacol.* 77, 228-236.

⁴Solt, L.A., *et al.* (2011) Suppression of TH17 differentiation and autoimmunity by a synthetic ROR ligand. *Nature* 472, 491-494.

II. Product Components & Storage Conditions

This Human ROR γ 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ ROR γ Reporter Cells	3 x 0.6 mL	-80°C
▪ Cell Recovery Medium (CRM)	2 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 45 mL	-20°C
▪ Ursolic Acid, 10 mM (in DMSO) (reference agonist for ROR γ)	1 x 30 μ L	-20°C
▪ Detection Substrate	3 x 2.0 mL	-80°C
▪ Detection Buffer	3 x 2.0 mL	-20°C
▪ Plate frame	1	ambient
▪ Snap-in, 8-well strips (white, sterile, collagen-coated wells)	12	-80°C

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 a 4-6 hr 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 **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 ROR γ Assay kit includes a 10 mM stock solution of Ursolic Acid, a reference inverse-agonist of human ROR γ . 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, 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.

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:* 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

Human RORγ Assay Products	
<i>Product No.</i>	<i>Product Descriptions</i>
IB04001-32	Human ROR γ Reporter Assay System 3x 32 assays in 96-well format
IB04001	Human ROR γ Reporter Assay System 1x 96-well format assay
IB04002	Human ROR γ Reporter Assay System 1x 384-well format assay
Mouse RORγ Assay Products	
M04001-32	Mouse ROR γ Reporter Assay System 3x 32 assays in 96-well format
M04001	Mouse ROR γ 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.	

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.

www.indigobiosciences.com

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 Ursolic Acid, and the setup of an RORγ dose-response assay.

