



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

96-well Format Assays
Product # IB04001

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Technical Manual
(version 7.1)

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Human ROR γ 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 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 principle application of this reporter 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, viability determinations, or cell titer adjustments.

▪ The Assay Chemistry ▪

INDIGO's nuclear receptor reporter assay format capitalizes 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. Do not use DMSO to further dilute test compound solutions. Immediately prior to setting up an assay, the master stocks are serially diluted using **Compound Screening Medium (CSM)** to achieve *2x-concentration* treatment media, as described in *Step 2* of this assay protocol. 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%.

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.

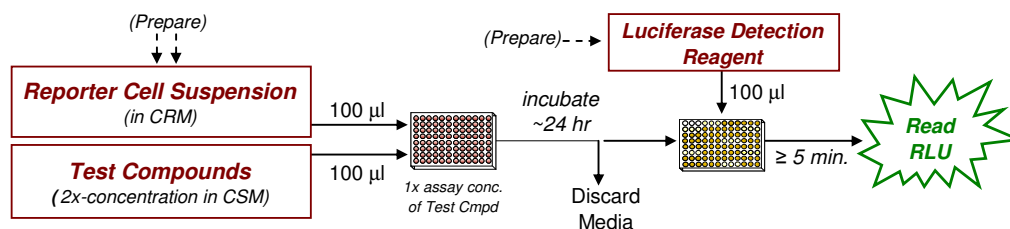
▪ Considerations for Automated Dispensing ▪

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 12 ml <i>(prepared from kit components)</i>	100 µl / well 9.6 ml / plate	~ 2.4 ml
LDR 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 are dispensed into wells of the assay plate and then immediately dosed with the user's test compounds. Following 22 -24 hr incubation, treatment media are discarded and prepared Luciferase Detection Reagent (LDR) 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 ▪

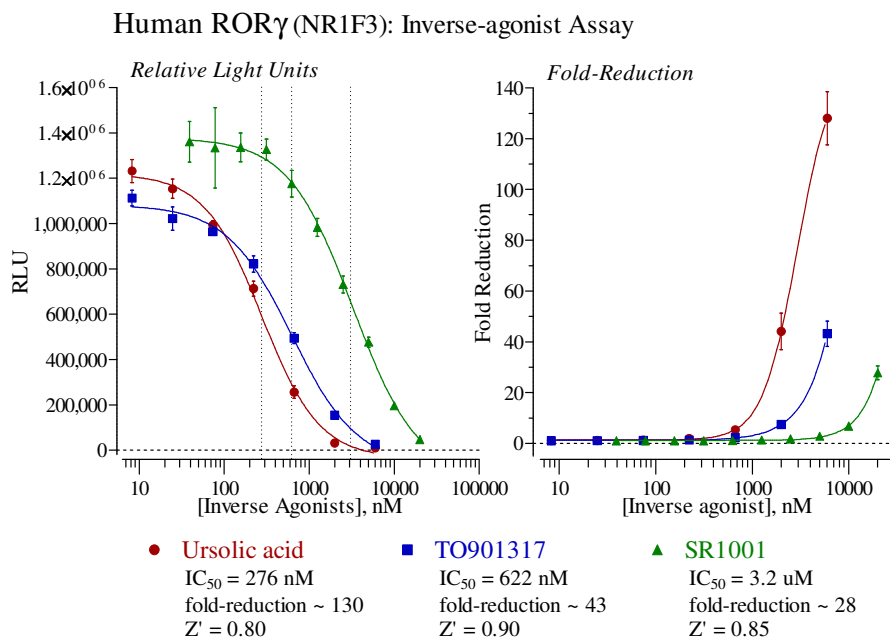


Figure 2. The Human ROR γ Assay.

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

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

The aliquot of 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.

<u>Kit Components</u>	<u>Amount</u>	<u>Storage Temp.</u>
▪ ROR γ Reporter Cells	1 x 2.0 mL	-80°C
▪ Cell Recovery Medium (CRM)	1 x 10.5 mL	-20°C
▪ Compound Screening Medium (CSM)	1 x 35 mL	-20°C
▪ Ursolic Acid, 10 mM (in DMSO) (reference agonist for ROR γ)	1 x 30 μ L	-20°C
▪ Detection Substrate	1 x 6.0 mL	-80°C
▪ Detection Buffer	1 x 6.0 mL	-20°C
▪ 96-well, <i>collagen-coated</i> assay plate (white, sterile, cell-culture ready)	1	-20°C

NOTE: This ROR γ 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% 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:* clear 96-well assay plate, sterile, 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 1-8* are performed on **Day 1**, requiring less than 2 hours to complete. *Steps 9-14* 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 Cell Recovery Medium (CRM) and Compound Screening Medium (CSM) from freezer storage, thaw and equilibrate to 37°C using a water bath.

2.) Prepare dilutions of treatment compounds (first see *Note 5.3*): Total DMSO carried over into assay reactions should never exceed 0.4%.

Note that, in *Step 6*, 100 µl of the prepared treatment media is added into assay wells that have been pre-dispensed with 100 µl of Reporter Cells. Hence, to achieve the desired *final* assay concentrations one must prepare treatment media with a 2x-concentration of the test and reference material(s). Plan dilution volumes carefully; this kit provides 35 ml of CSM.

Preparing the positive control: This ROR γ Reporter Assay kit includes a 10 mM stock solution of Ursolic Acid, an inverse-agonist of ROR γ that may be used as a reference. 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.

3.) Rapid Thaw of the Reporter Cells: *First*, retrieve the tube of CRM from the 37°C water bath and sanitize the outside surface 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 10 ml volume of the 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 12 ml.

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

5.) Gently invert the tube of Reporter Cells several times to disperse cell aggregates and gain an homogenous cell suspension. Transfer the cell suspension into a reservoir and, using an 8-channel pipette, dispense 100 µl of cell suspension into each well of the assay plate.

NOTE 5.1: 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 5.2: 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 (100 µl/well) into a clear 96-well cell culture treated assay plate, followed by 100 µl/well of CSM. Incubated overnight in identical manner to those reporter cells contained in the white assay plate.

NOTE 5.3: For logistical reasons, some users find it more convenient to first plate the reporter cells and then prepare their test compound dilutions. That strategy works equally well. Once plated, cells may be placed in an incubator for up to 3 hours before proceeding to *Step 6*.

6.) Dispense 100 µl of 2x-concentration treatment media into appropriate assay wells.

7.) Transfer the assay plate into a 37°C, humidified 5% CO₂ incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity ($\geq 85\%$) environment within the cell culture incubator. This is critical to prevent the onset of deleterious "edge-effects" in the assay plate.

8.) 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.

9.) 30 minutes before intending to quantify ROR γ 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. 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.

10.) 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*.

11.) *Immediately before proceeding to Step 12*, 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.

12.) Following 22 - 24 hours of incubation 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.

13.) Add 100 μ l of **LDR** to each well of the assay plate. Allow the assay plate to rest at room temperature for at least 5 minutes. Do not shake the assay plate during this period.

14.) 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
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.

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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.

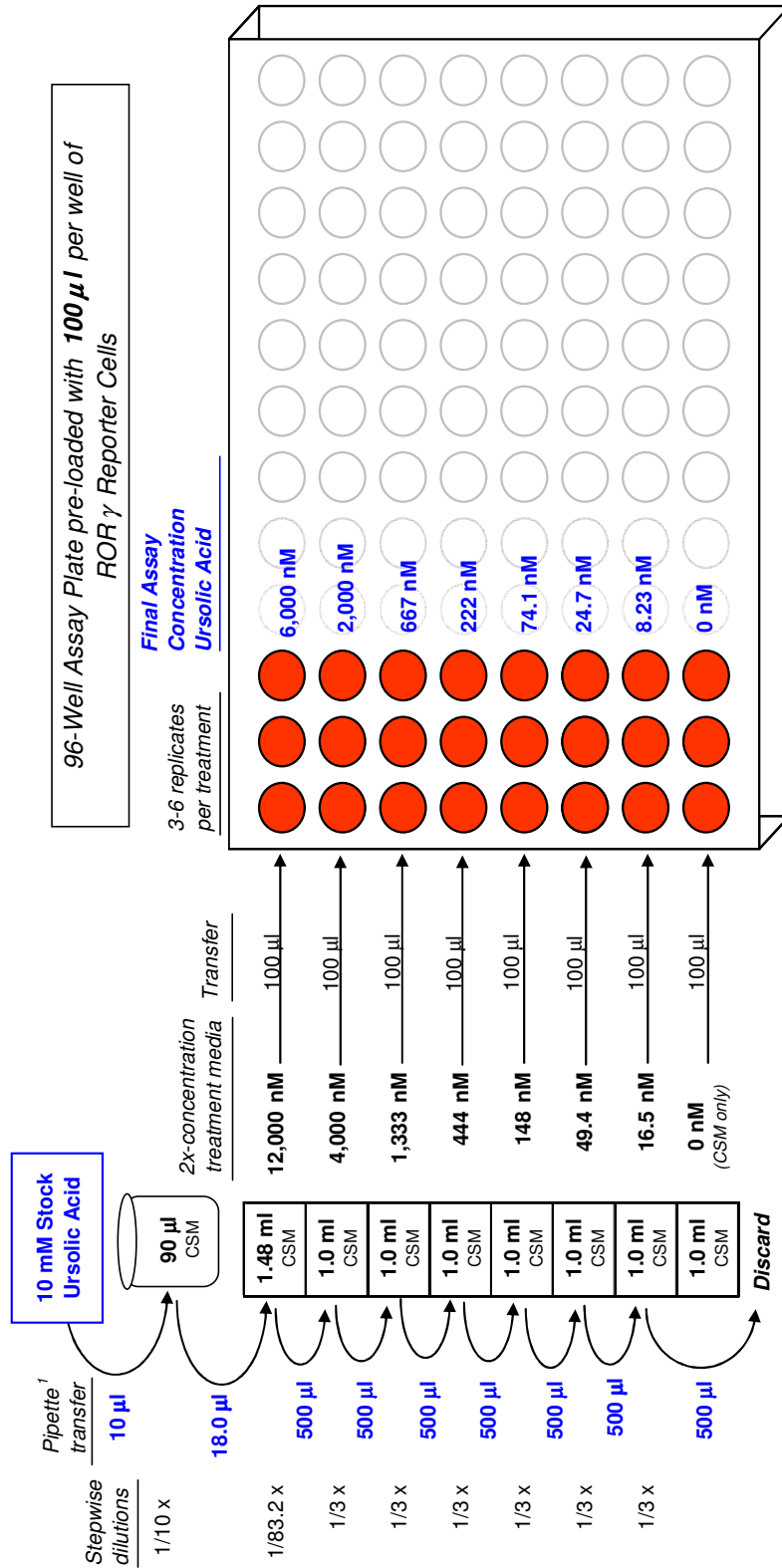
“CryoMite” is a Trademark TM of INDIGO Biosciences, Inc. (State College, PA)

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

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APPENDIX 1

Example scheme for the serial dilution of the inverse-agonist Ursolic Acid, and the setup of an ROR γ 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.