Mouse RAR-related Orphan Receptor, Gamma
(nr1f3, rorC, mRORγ)
Reporter Assay System

3x 32 Assays in 96-well Format
Product # M04001-32

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Technical Manual
(version 7.2i)

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Mouse RORγ Reporter Assay System

3x 32 Assays in 96-well Format

I. Description

▪ The Assay System ▪

This nuclear receptor assay utilizes proprietary human cells engineered to provide high-level expression of a hybrid form of the **Mouse RORγ** (RAR-related Orphan Receptor Gamma; nr1f3) referred to as mouse rorC, or here as **mRORγ**. The N-terminal DNA binding domain (DBD) of the native mRORγ receptor has been substituted with that of the yeast GAL4-DBD.

As is true *in vivo*, these reporter cells express mRORγ in a constant state of high-level activity. **Figure 2** demonstrates the constitutive activity of mRORγ in the absence of ligand treatments. 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 mouse RORγ.

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 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 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 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 agonist, **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.

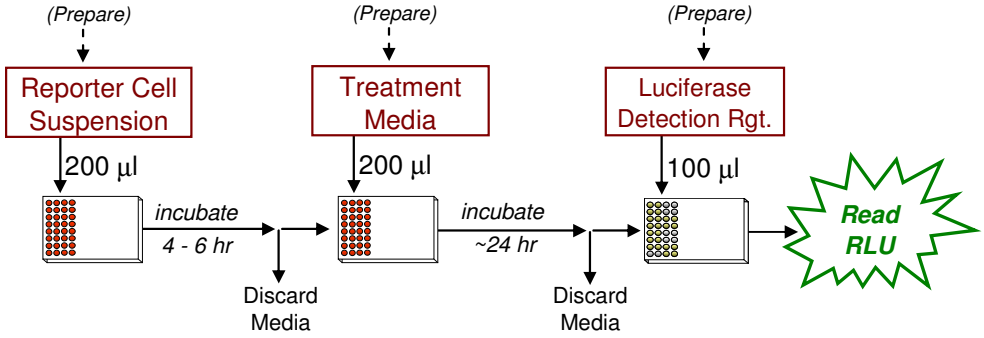
Alternatively, if test compound solubility is expected to be problematic,
2.) DMSO may be used to make serial dilutions, thereby generating 1,000x-concentrated stocks for each independent test concentration. Treatment media are then prepared using CSM to make final 1,000-fold dilutions of the prepared 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%. 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 are considered to be 'single-use' reagents.

▪ Assay Scheme ▪

Figure 1. Assay workflow. *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 hours incubation, treatment media are discarded, and Luciferase Detection Reagent is added. The intensity of light emission (in terms of 'Relative Light Units'; RLU) from each assay well is quantified using a plate-reading luminometer.



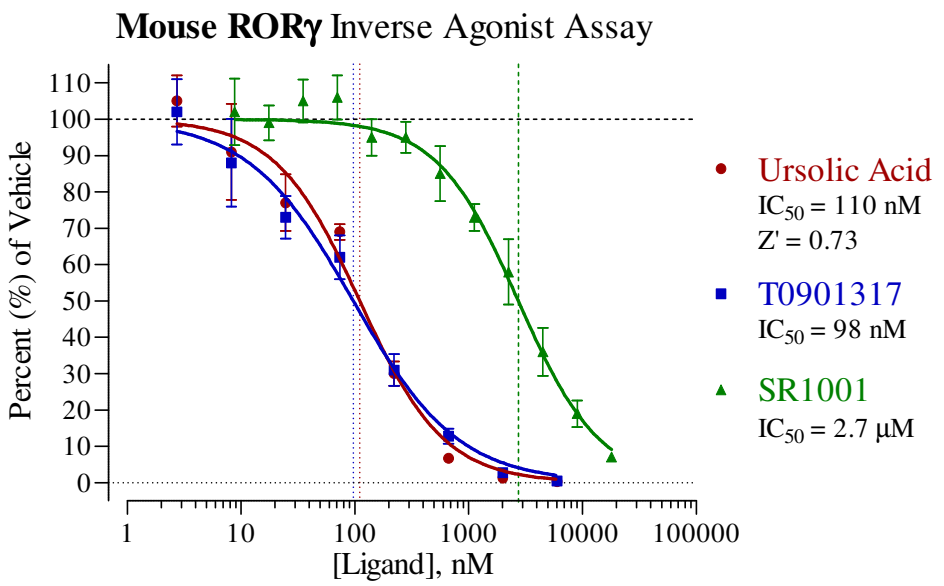


Figure 2. The Mouse RORγ Assay.

Dose-response analyses of the mouse RORγ Assay were performed using the inverse-agonists Ursolic Acid[2] (provided), T0901317[3] (Tocris), and SR1001[4] (Cayman Chemical). 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 percent activity relative to the DMSO treated cells and respective values of percent coefficient of variation (%CV) were determined for each treatment concentration. Non-linear regression analyses were performed and IC_{50} values determined using GraphPad Prism software.

Results: Ursolic acid demonstrates dose-dependent reduction of mRORγ with an $IC_{50} \leq 110$ nM. The large assay window and corresponding high Z' value[1] confirm the robust performance of this assay.

[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*($SD^{Vehicle}$ + $SD^{Reference\ max.}$) / ($RLU^{Vehicle}$ – $RLU^{Reference\ max.}$)]

[2]Xu, 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.

[3]Kumar, N., *et. al.* (2010) The benzenesulfoamide T0901317 [N-(2,2,2-trifuoroethyl)-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.

[4]Solt, 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 mouse mRORγ 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.

Reporter cells are temperature sensitive! To ensure maximal viability the tube of Reporter 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.

Kit Components	*Amount*	*Storage Temp.*
▪ Mouse RORγ 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 mRORγ)	1 x 30 µL	-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

▪ dry ice bucket (*Step 2*)
▪ 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-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 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 tubes of **Reporter Cells** from -80°C storage and place them directly into dry ice for transport to the laminar flow hood: 1 tube for 32 assay wells, 2 tubes for 64 assay wells, or 3 tubes for 96 assay wells. When ready to begin, transfer the tube(s) of reporter cells into a rack and, *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.

Third, during the 5 - 10 minutes incubation period, work in the cell culture hood to *carefully* mount four sterile 8-well strips into the blank assay plate frame. Strip-wells are fragile. Note that they have keyed ends (square and round), hence, they will fit into the plate frame in only one orientation.

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 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, ≥ 70% 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.) 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 not exceed 0.4%.

b. Prepare the positive control: This kit includes a 10 mM stock solution of Ursolic Acid, an inverse-agonist of mRORγ 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.

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 either a single-tip or 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% CO_2 incubator for 22 - 24 hours.

NOTE: Ensure a high-humidity (≥ 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 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.

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

Product No.	*Product Descriptions*
Human RORγ Assays	
IB04001-32	Human RORγ Reporter Assay System 3x 32 assays; 8-well strips in 96-well format plate frame
IB04001	Human RORγ Reporter Assay System 1x 96-well format assay
IB04002	Human RORγ Reporter Assay System 1x 384-well format assay
Mouse RORγ Assays	
M04001-32	Mouse RORγ Reporter Assay System 3x 32 assays; 8-well strips in 96-well format plate frame
M04001	Mouse RORγ Reporter Assay System 1x 96-well format assay
Rat RORγ Assays	
R04001-32	Rat RORγ Reporter Assay System 3x 32 assays; 8-well strips in 96-well format plate frame
R04001	Rat RORγ 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	
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 contained in 5 x 96-well assay plates
LCM-10	Reagent in **10x bulk volume** to perform **960** Live Cell Assays contained in 10 x 96-well assay plates
INDIGlo Luciferase Detection Reagent	
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

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

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

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.

APPENDIX 1

Example scheme for the serial dilution of the inverse-agonist Ursolic Acid, and the setup of a mouse RORγ dose-response assay.